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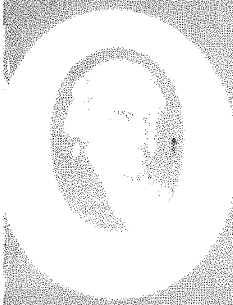

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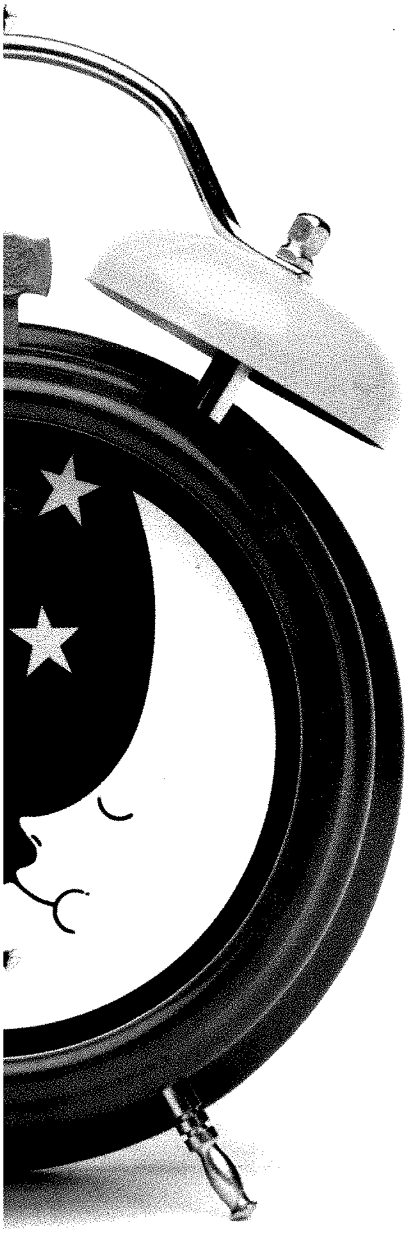

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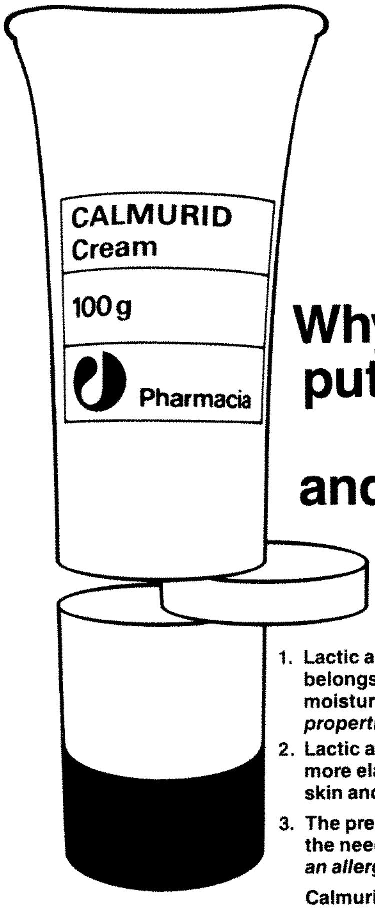

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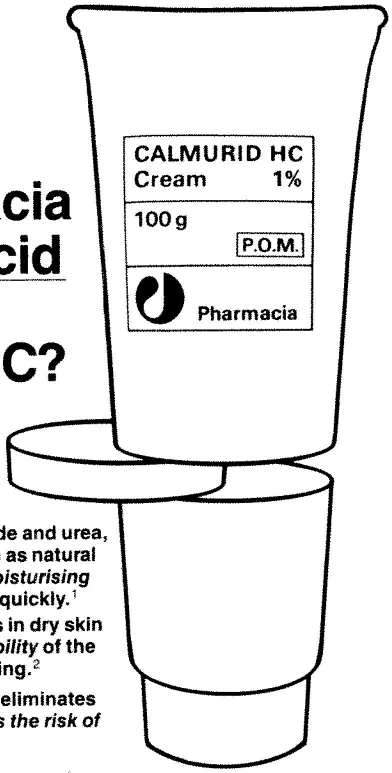

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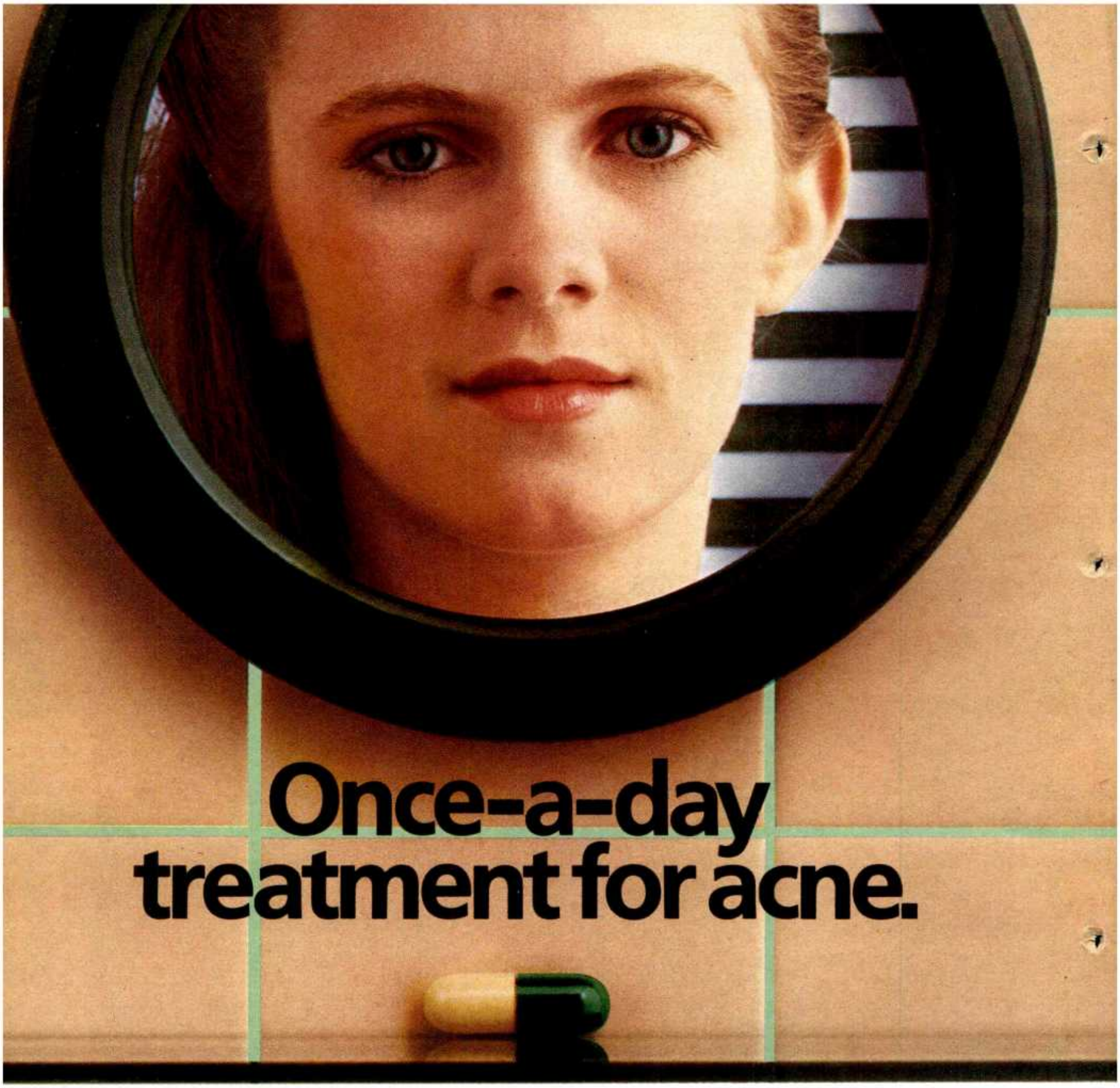
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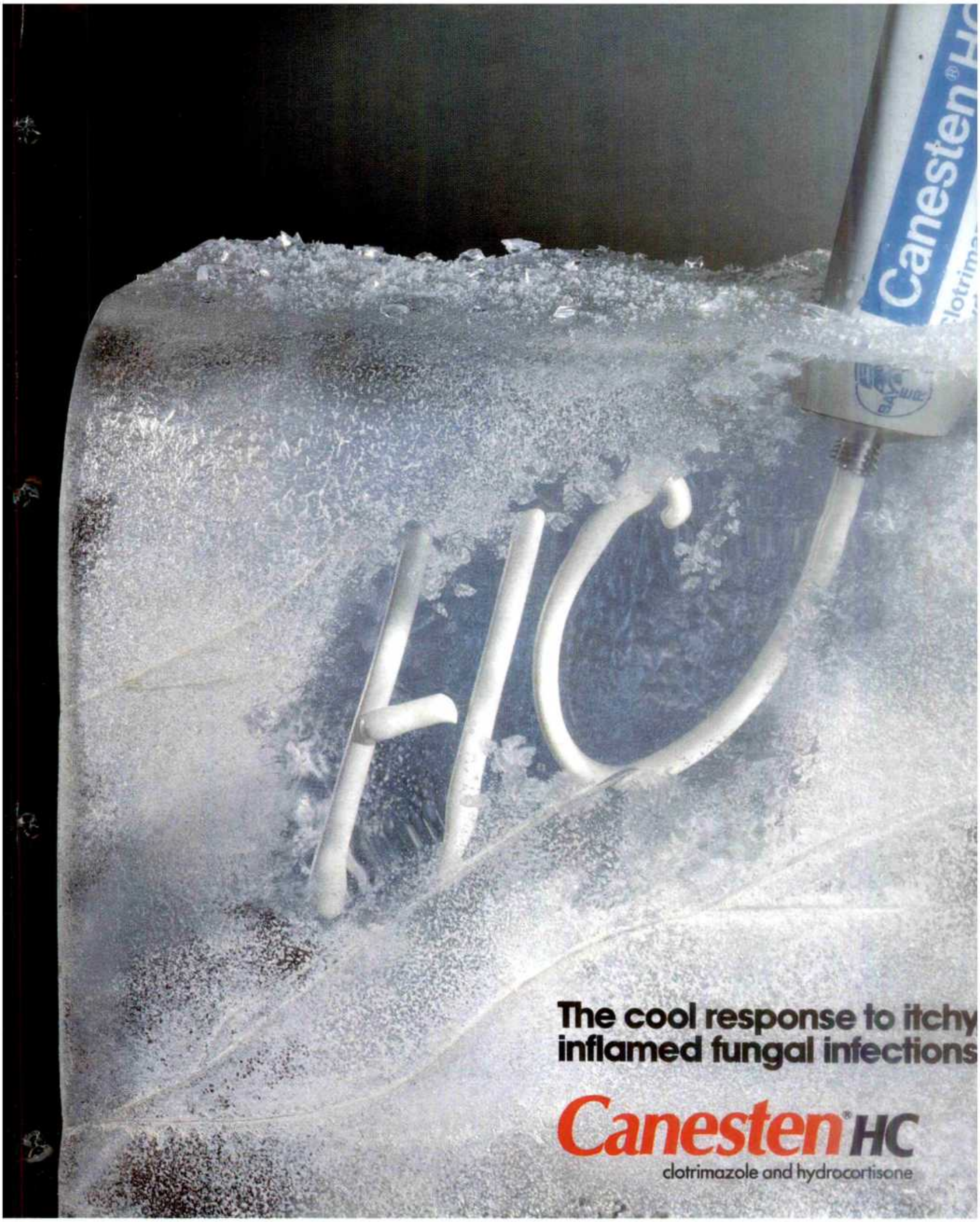
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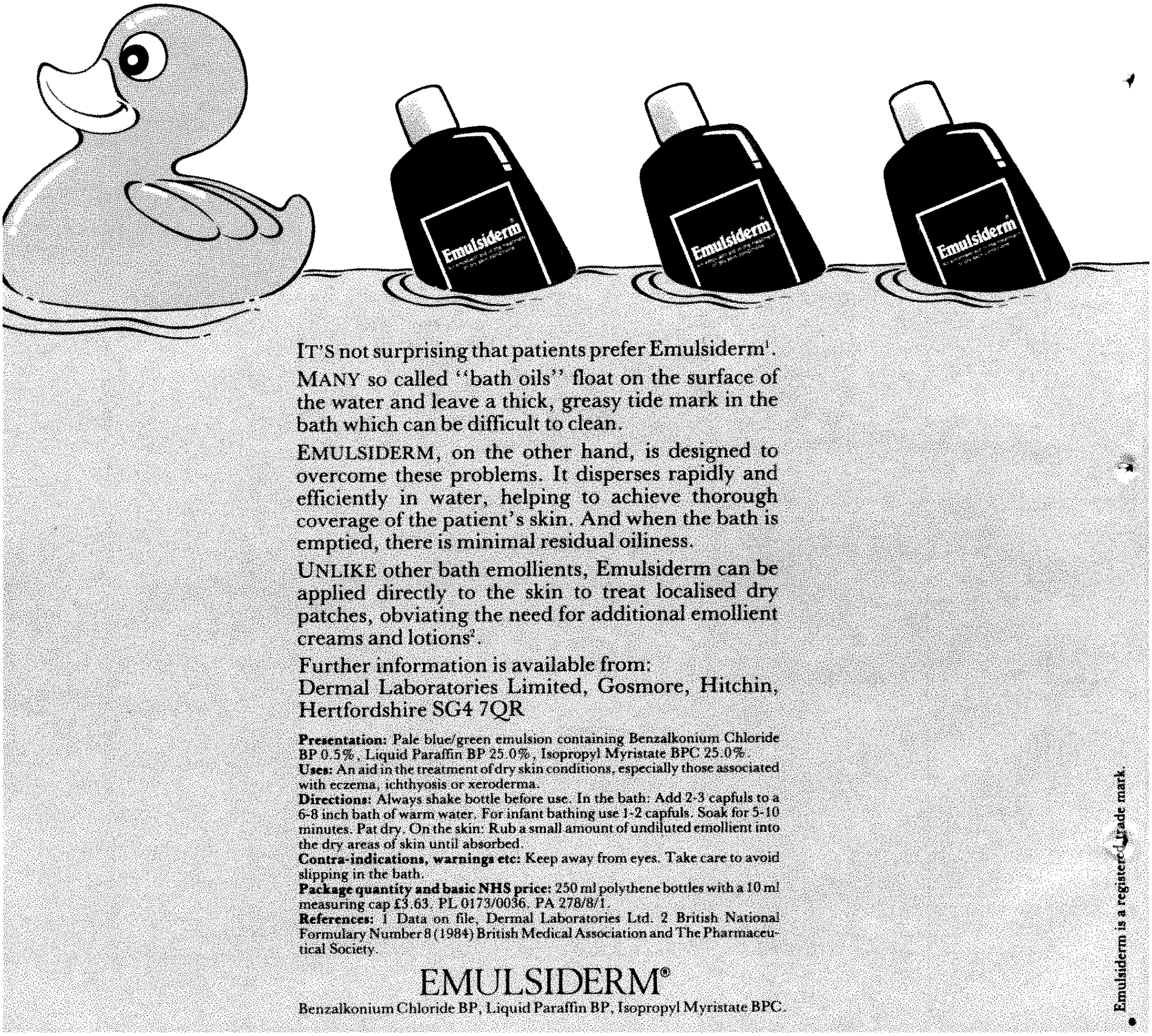

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*British Journal of Dermatology* (1986) **114**, 1–6.

# Demonstration of anaphylatoxins C3a, C4a and C5a in the scales of psoriasis and inflammatory pustular dermatoses

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Accepted for publication 22 May 1985

## SUMMARY

Complement components C3a, C4a and C5a were assayed in corneal scale extracts from psoriasis and other dermatoses characterized by sterile subcorneal pustules, using radioimmunoassay. Larger amounts were detected in psoriasis and related pustular dermatoses than in extracts of non-inflammatory stratum corneum. It is concluded that complement is activated via the classical pathway and releases the neutrophil chemotactic fragment C5a.

Polymorphonuclear leukocytes (PMN) often migrate individually through breaks in the basement membrane into the suprapapillary plate of the lesional epidermis of psoriasis. Their focal accumulation in the subcorneal layer or in the parakeratotic mound, known as a microabscess, is a consistent histological finding in psoriasis and related pustular dermatoses (Pinkus & Mehregan, 1966). Tagami & Ofuji (1977) demonstrated that psoriatic scale contained unique PMN-chemotactic factor(s), which they suggested were derived from complement. Three different mechanisms have been proposed for the generation of chemotactic fragments of complement in psoriatic lesions; complement activation through the classical or alternative pathway (Beutner *et al.*, 1978; Kaneko *et al.* 1980; Marley *et al.*, 1982) or direct cleavage of a component of complement by serine protease (Lazarus, Yost & Thomas, 1977).

When complement is activated through the classical pathway, both C4a and C3a anaphylatoxins are released, while only C3a is released in the case of the alternative pathway. In direct enzymatic cleavage of C5, no C3a or C4a generation occurs.

We have reported previously increased levels of C3a and C4a in psoriatic sera (Ohkohchi, Takematsu & Tagami, 1985). We have now shown that scale extracts from psoriasis and sterile pustular dermatoses contain increased amounts of C5a, C3a and C4a compared with non-inflammatory skin.

Correspondence: Dr H. Tagami, Department of Dermatology, Tohoku University School of Medicine, 1-1 Seiryo-machi, Sendai 980, Japan.

## METHODS

Scales were collected from skin lesions in 14 patients with psoriasis vulgaris, four with psoriatic erythroderma, nine with pustular psoriasis and eight with pustulosis palmaris et plantaris (PPP), and from non-inflammatory skin from seven patients including three with tylosis, one clavus, one X-linked ichthyosis and two normal skin. Scales were also collected from one patient with subcorneal pustular dermatosis (SPD) and one with pityriasis rubra pilaris (PRP). The scales were stored at −70°C.

### *Extracts of stratum corneum*

Water-soluble components were extracted from scales in phosphate-buffered saline pH 7·4 containing streptomycin 100 μg/ml, as reported previously (Tagami & Ofuji, 1977).

### *Radioimmunoassay*

Radioimmunoassays for human C3a and C3a des arginine, C4a and C4a des arginine, and C5a and C5a des arginine, referred to here collectively as C3a, C4a and C5a, were performed as reported previously (Ohkohchi *et al.*, 1985).

### *Statistical analysis*

Levels of anaphylatoxins were compared using the Wilcoxon rank-sum test and pooled anaphylatoxin levels compared using Spearman's rank correlation coefficient.

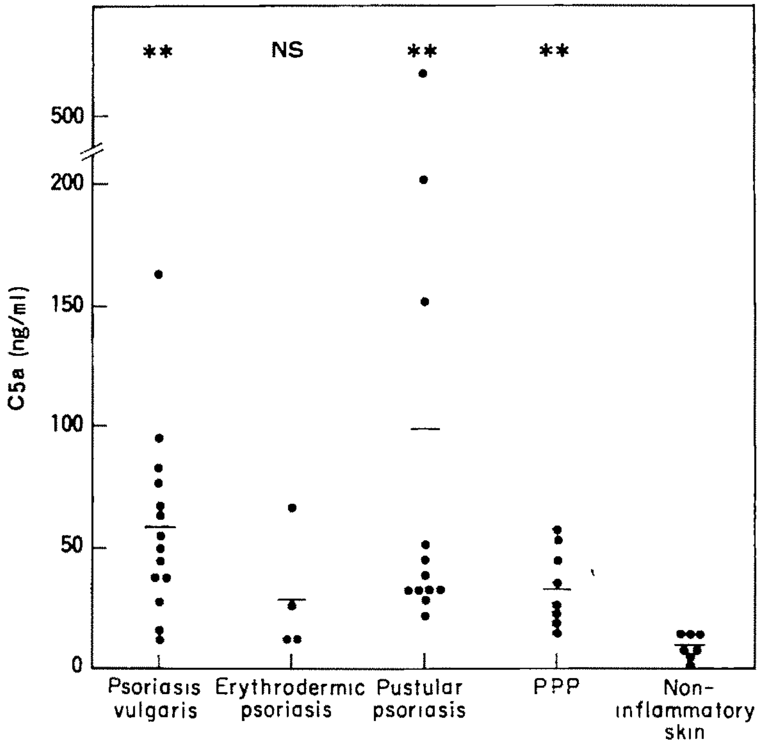

FIGURE 1. Levels of C5a in scale extracts from psoriasis vulgaris, erythrodermic psoriasis, pustular psoriasis, PPP, and non-inflammatory skin. Bars represent means. Comparison with non-inflammatory skin: *$P<0{\cdot}005$, **$P<0{\cdot}001$, n.s., not significant.

## RESULTS

### *Anaphylatoxin levels in scale extracts*

In comparison with non-inflammatory skin, levels of C3a, C4a and C5a were significantly elevated in scale extracts from psoriasis vulgaris, pustular psoriasis and PPP, but not in erythrodermic psoriasis (Figs 1–3). Taking the mean ± 2 S.D. for the controls as the normal range, C3a levels, however, were elevated in three out of four cases of erythrodermic psoriasis. In SPD, levels of all three anaphylatoxins were higher than in the controls, while in PRP the levels all fell within the normal range.

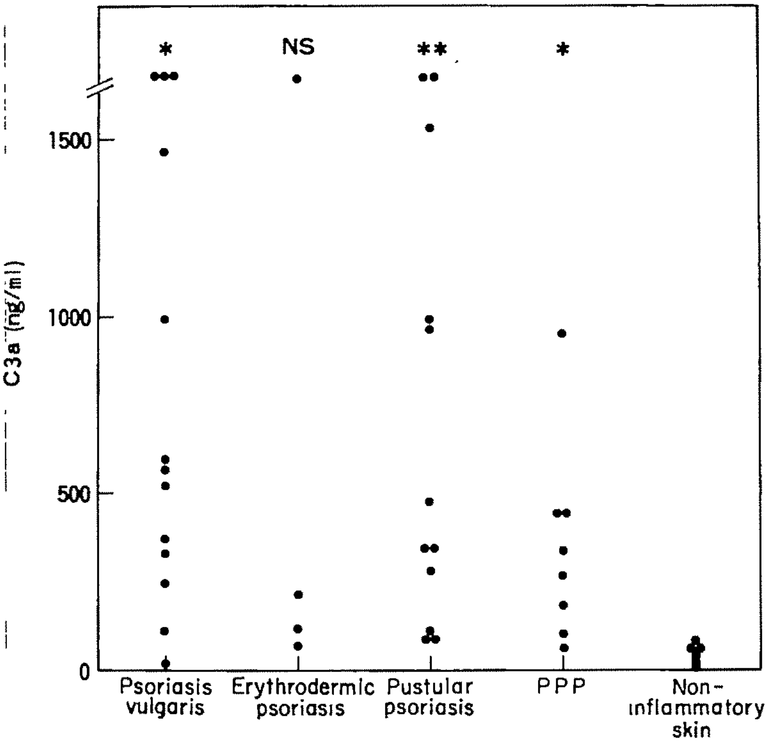

FIGURE 2. Levels of C3a in scale extracts from psoriasis vulgaris, erythrodermic psoriasis, pustular psoriasis, PPP, and non-inflammatory skin. Comparison with non-inflammatory skin: $*P < 0.005$, $**P < 0.001$, n.s., not significant.

### *Correlation between levels of different anaphylatoxins*

When the anaphylatoxin levels were pooled, there were significant positive correlations between the levels of all three:

C3a and C4a $r_s = 0.95$ $P < 0.001$
C3a and C5a $r_s = 0.88$ $P < 0.001$
C4a and C5a $r_s = 0.87$ $P < 0.001$.

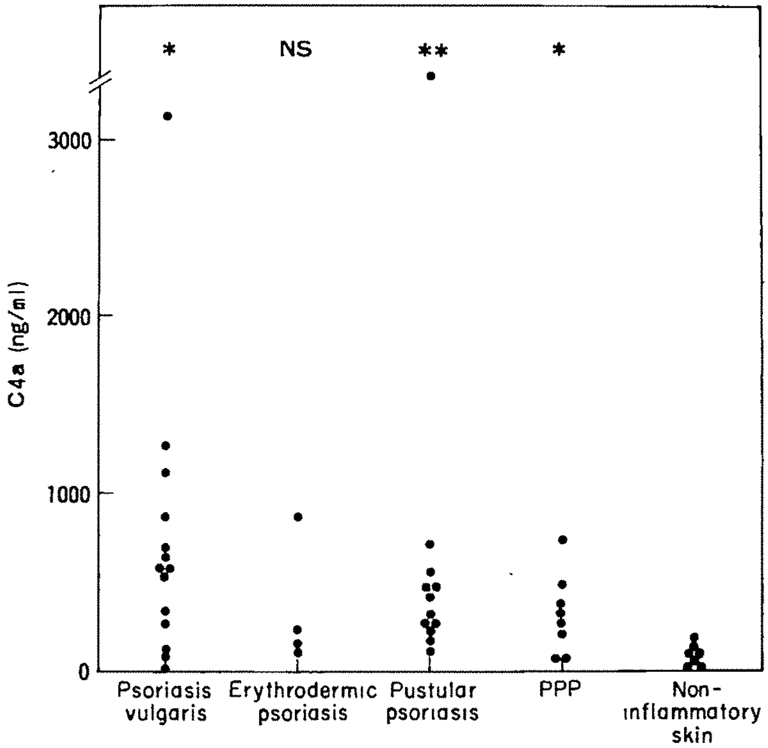

FIGURE 3. Levels of C4a in scale extracts from psoriasis vulgaris, erythrodermic psoriasis, pustular psoriasis, PPP, and non-inflammatory skin. Comparison with non-inflammatory skin: $^{*}P<0{\cdot}005$, $^{**}P<0{\cdot}001$, n.s., not significant.

## DISCUSSION

Our results demonstrate that the scales of psoriasis and related sterile pustular dermatoses contain high levels of anaphylatoxins.

In addition to C5a, mol. wt. 11,000 (Fernandez & Hugli, 1978), psoriatic scales contain several other potentially chemotactic factors such as bacterial chemotactic factors, denatured proteins, PMN themselves (Dahl, Lindroos & Nelson, 1978; Tagami *et al.*, 1982), and leukotriene $B_4$ (Grabbe, Czarnetzki & Mardin, 1982; Brain *et al.*, 1983). Since a PMN chemotactic factor mol. wt. approximately 12,000 is consistently found in scale extracts from psoriasis, pustular psoriasis, PPP, SPD and eosinophilic pustular folliculitis (Tagami & Ofuji, 1977, 1978; Tagami *et al.*, 1982, 1983; Takematsu & Tagami, 1986), it is possible that C5a is an important chemoattractant in these lesions. However, treatment of this chemotactic factor with anti-C5a antiserum only partially abrogated the chemotactic activity (H. Takematsu & H. Tagami, unpublished data), suggesting the presence of other chemotactic factors of similar molecular weight, such as epidermal cell thymocyte-activating factor (Luger *et al.*, 1983).

There remains controversy as to which complement activation pathway is involved in the generation of C5a. The measurement of anaphylatoxins, as in our previous study (Ohkohchi *et al.*, 1985) allows an assessment of complement activation in lesional skin (Hugli & Chenoweth, 1980). We have demonstrated that scale extracts obtained from psoriasis and related pustular dermatoses contained significantly higher levels of C3a and C4a than non-inflammatory skin and that the levels of the different anaphylatoxins were positively correlated, suggesting that activation of complement occurred chiefly through the classical pathway. There is also the possibility of direct generation of C5a by proteinases, which are found to be increased in the upper portion of the psoriatic epidermis (Lazarus *et al.*, 1977; Dubertret *et al.*, 1982). Direct enzymatic cleavage of C5 in lesional skin could proceed without complement activation. The results of the present study agree with our previous report of increased serum levels of C3a and C4a in psoriatic patients. These levels decreased after successful treatment of the skin lesions (Ohkohchi *et al.*, 1985).

The low anaphylatoxin levels found in erythrodermic psoriasis suggest that complement activation in the lesional skin may not be an important feature in this type of psoriasis.

PRP is occasionally difficult to differentiate from psoriasis clinically and histologically (Davidson, Winkelmann & Kierland, 1969) because of the inflammatory scaly appearance and increased epidermopoiesis. The lesions, however, lack the transepidermal migration of PMN which is characteristic of psoriasis. We found low levels of anaphylatoxins in scale extracts from PRP, within the normal range found in non-inflammatory skin.

## ACKNOWLEDGMENTS

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# Cutaneous immunological studies in diagnosis of acute graft-versus-host disease

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## SUMMARY

A comparative study of the healthy skin of patients who had undergone bone marrow grafting and not developed graft-versus-host disease (GVHD) and of patients with cutaneous lesions of acute GVHD has been carried out. The aim of this study was to assess the diagnostic value of cutaneous immunopathology in the diagnosis of acute GVHD. A double-labelling immunofluorescence technique was used with a panel of monoclonal antibodies. The results showed a lack of specificity for GVHD in the distribution of Langerhans cells, but confirmed the diagnostic value of HLA-DR staining of epidermal keratinocytes. Cellular polymorphism of the T cell infiltrate in the dermis was observed (T helpers 40% and T suppressors 20%). The expression of the 55–57 Kd keratin polypeptide and of bullous pemphigoid antigen showed modification during acute GVHD while that of pemphigus antigen remained unchanged.

Immunological studies carried out on cutaneous lesions of acute GVHD have demonstrated changes in various cell populations. Suitters & Lampert (1983) have reported loss of OKT6 and Ia staining on Langerhans cells, while Lampert, Suitters & Chisholm (1981), and Mason, Dallman & Barclay (1981) have reported the appearance of Ia staining on epidermal keratinocytes. It has been suggested that changes in the proportion of T helper and T suppressor cells in the dermal cell infiltrate may also be of value in diagnosis (Lampert *et al.*, 1982).

It is clearly of importance to establish that these changes seen in patients with frank GVHD are associated with the disease process, and not with the prior intensive chemotherapy and total body irradiation which precedes marrow grafting. For this reason, we have carried out cutaneous immunopathological studies on apparently normal skin from patients who had received bone marrow grafts and compared changes observed in these biopsies with skin biopsies from patients with acute GVHD. We have also studied in detail the expression of the 55–57 Kd keratin polypeptides in these two groups of patients and the expression of pemphigus antigen and bullous pemphigoid antigen.

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## METHODS

### *Patients*

Fourteen patients were studied in all. Eleven of these patients were receiving bone marrow allografts and three autografts (Table 1).

All patients were biopsied routinely on days 15 and 30. In addition to this, six of the eleven allograft patients and two of the three autograft patients were biopsied on day 100. Six of the eleven allograft patients developed acute graft-versus-host disease. This was graded clinically according to Thomas's classification (Thomas *et al.*, 1985). Two patients had grade II disease, three had grade III and one grade IV.

The 15-day biopsy was carried out on 11 patients with normal skin and three patients with skin lesions. Biopsies carried out on day 30 were from six patients with skin lesions and eight patients with normal skin. The biopsies at day 100 were from five cases with skin lesions and three with normal skin. In addition, four totally normal control subjects without skin lesions or any haematological problems underwent skin biopsy.

The histological appearance of the abnormal skin biopsies were graded according to Lerner's classification (Lerner *et al.*, 1974). One patient had a grade I disease (vacuolar degeneration of basal cells). Two biopsies showed evidence of grade II histological changes (exocytosis and satellite cell necrosis), two patients had evidence of grade III changes (perivascular infiltration of the superficial and middle dermis) and one patient had histological evidence of grade IV disease (papillary oedema, parakeratosis and intense keratinocyte necrosis).

Of the 11 patients who received allografts, nine had acute leukemia and two had chronic myeloid leukemia. The three patients receiving autografts had acute leukemia. Prior to bone marrow transplantation the chemotherapy regime was as follows: nine patients received cyclophosphamide 120 mg/kg; three patients received carmustine 300 $mg/m^2$, etoposide 300 $mg/m^2$ and cyclophosphamide 120 mg/kg; two patients received BCNU 300 $mg/m^2$, etoposide 300 $mg/m^2$, cytosine arabinoside 300 $mg/m^2$ and cyclophosphamide 120 mg/kg. These varying chemotherapeutic regimes were followed the day prior to transplantation by whole body irradiation of 10 Gy in a single exposure. The allografted patients received $2–4 \times 10^8$ nucleated cells/kg from a mixed lymphocyte culture-identical donor. The autografted patients had their bone marrow purged using Asta-Z. An attempt was made to prevent graft-versus-host disease by giving methotrexate 15 $mg/m^2$ on the day prior to grafting and then 10 $mg/m^2$ on days 3, 6, 11 and 18. This was then followed by 20 $mg/m^2$ of methotrexate intravenously and 12 mg of methotrexate intrathecally, given on alternate weeks until day 100. In addition, cyclosporin A

TABLE 1. Patients studied

| | | Number of patients biopsied | | |
|---|---|---|---|---|
| | Haematological disorder | Day 15 | Day 30 | Day 100 |
| Allograft<br>(11 cases) | acute leukemia<br>(9 cases) | 9 | 9 | 4 |
| | chronic myeloid leukemia<br>(2 cases) | 2 | 2 | 2 |
| Autograft<br>(3 cases) | acute leukemia<br>(3 cases) | 3 | 3 | 2 |

3 mg/kg per day was given intravenously continuously from days 1 to 15 and then reduced to 12 mg/kg per day given orally in two divided doses depending on tolerance, until day 100.

Four millimetre punch biopsies were taken and frozen in liquid nitrogen. Sections (3 $\mu$m) were cut and immersed for 10 min in acetone, and thereafter washed in PBS for 10 min. The monoclonal antibodies used are detailed in Table 2 with appropriate dilutions. Monoclonal antibody KL1 which is a marker for the 55–57 kd keratin polypeptides was used in addition, as was serum from patients with pemphigus vulgaris and bullous pemphigoid.

TABLE 2. Immunological reagents used

| Reagent | Dilution | Target cells |
|---|---|---|
| $IOT_1$ | $\frac{1}{5}$ | peripheral T lymphocytes (Pan T) |
| $IOT_4$ | $\frac{1}{5}$ | inducer cells |
| $Leu_3$ | | |
| $IOT_8$ | $\frac{1}{5}$ | T suppressor/cytotoxic cells |
| $Leu_2$ | | |
| $IOT_6$ | $\frac{1}{5}$ | thymocyte antigen and epidermal Langerhans cells |
| $OKT_{10}$ | $\frac{1}{10}$ | activated T cells |
| $IOB_1$ | $\frac{1}{5}$ | peripheral B cells |
| $IOT_2$ | $\frac{1}{10}$ | HLA DR antigens |
| $KL_1$ | | marker for keratin polypeptides (55–57kd) |
| Pemphigus serum of patients | | |
| Pemphigoid serum of patients | | |

After 30 min incubation at 37°C, the sections were washed in PBS, then fluorescein-conjugated goat anti-mouse IgG (Meloy) diluted 1/5 was applied for 30 min at 37°C followed by washing in PBS. For the double-labelling study, the sections were then incubated with phycoerythrin-conjugated Leu 2 or Leu 3 antibodies (Becton Dickinson) in darkness at room temperature after the sites had been saturated with normal mouse serum; the sections were then washed for 30 min in darkness before being mounted using buffered glycerin.

The sections were examined at × 320 magnification in a Zeiss microscope with two excitation filters (fluorochrome and rhodamine). Thus, by using the double-labelling technique, the percentage of T helper and T suppressor cells compared with the total number of HLA-DR+ cells can be determined by the following formulae:

$$\text{T suppressor cells} = \frac{\text{Leu}^2+\text{ cells}}{\text{Total HLA-DR}+\text{ cells}} \times 100$$

$$\text{T helper cells} = \frac{\text{Leu}^3+\text{ cells}}{\text{Total HLA-DR}+\text{ cells}} \times 100$$

In addition, the number of epidermal dendritic cells stained with IOT6 was determined in each patient on one section and compared with four samples of normal skin.

## RESULTS

### *Langerhans cells*

Normal skin, biopsied on day 5, showed a decrease in HLA-DR (IOT2) staining of Langerhans cells (2–3 cells/field) in the epidermis, which persisted until day 30. The reduction in Langerhans cell numbers was even more pronounced (0–2 cells/field) in the epidermis of acute GVHD lesions biopsied on days 15 and 30.

In eight biopsies performed on day 100, three patients (two with a history of acute GVHD and one without GVHD) showed IOT6+ and HLA-DR+ (IOT2) Langerhans cells in the epidermis (2–5 cells/field). None of these patients developed chronic GVHD.

Five patients (four with a history of acute GVHD and one with no lesions) still had no Langerhans cells in the epidermis at day 100. Three of these five patients developed chronic GVHD. The mean number of Langerhans cells in control sections was 5–10 cells/field.

### *Infiltrate*

In the normal control skin no infiltrate was found. In the dermis in grafted patients in the absence of cutaneous lesions at days 15 and 30, there was a mild infiltrate in the dermis (5–10 cells/field) made up essentially of IOT2+ (HLA-DR) cells for which double-labelling showed only 10% of IOT2+ cells with Leu-3 receptors. B lymphocytes and T suppressors (IOT8) were absent, the OKT10 marker was negative and the dermal vessels were Ia+.

In acute GVHD lesions at day 14 and 30, an infiltrate of moderate intensity (10–20 cells/field) was found in the papillary and reticular dermis. In the papillary dermis the cells lay along the basal membrane. These cells were all IOT2+ and IOT1+, and double-labelling showed that 40% of IOT2+ cells had Leu-3 (T-helper) receptors and 20% Leu-2 (T-suppressor).

There was intense epidermotropism, with cells presenting either an IOT2+ phenotype alone or IOT2+, Leu-2+ phenotype. In contrast with findings for the dermis, there were no T-helper cells in the epidermis. The dermal vessels showed HLA-DR staining; B lymphocytes were absent and no OKT10 positive cells were seen.

### *Keratinocytes*

In acute GVHD lesions, HLA-DR staining of keratinocytes was found (Table 3), whereas none was detected in biopsies of patients without GVHD. This staining was mainly in the basal and suprabasal layers (Fig. 1), was essentialy peripheral, similar in appearance to that found in pemphigus and was proportional to the severity of GVHD. HLA-DR staining was found in all six of the patients with acute GVHD.

TABLE 3. HLA-DR staining in graft versus host disease

| | Day 15 | | Day 30 | | Day 100 | |
|---|---|---|---|---|---|---|
| GVHD Case No. | keratinocytes HLA-DR+ | cutaneous lesions | keratinocytes HLA-DR+ | cutaneous lesions | keratinocytes HLA-DR+ | cutaneous lesions |
| 1 | − | ± | + | + | − | − |
| 2 | − | ± | + | + | − | + |
| 3 | − | − | − | + | + | + |
| 4 | + | + | − | + | + | + |
| 5 | − | − | + | + | − | + |
| 6 | − | − | + | + | − | + |

Appearance of HLA-DR staining is not an early marker of GVHD; it appears at the same time as cutaneous lesions or later

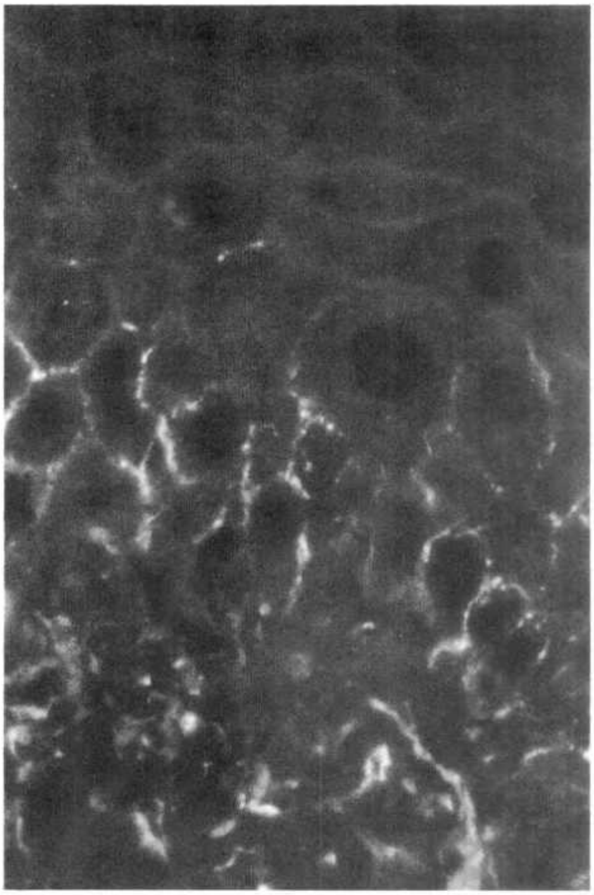

FIGURE 1. HLA-DR staining of keratinocytes was mainly in the suprabasal layers.

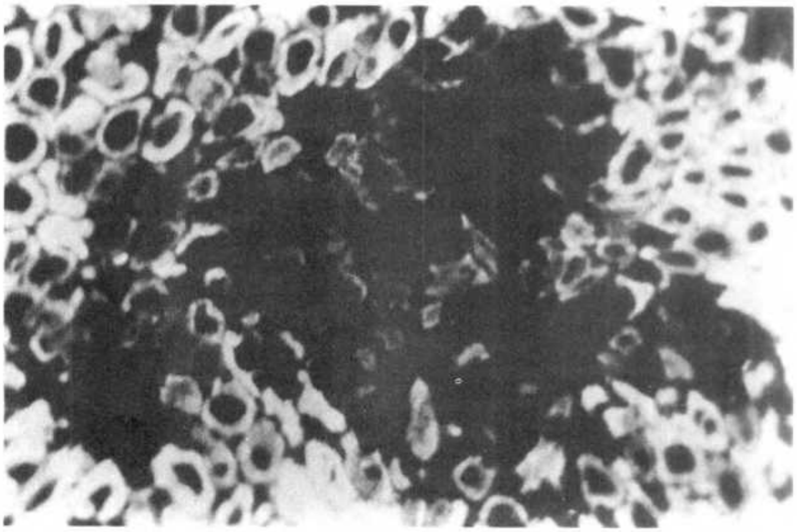

FIGURE 2. Expression of keratin polypeptides (55–57 kd) disappeared in certain areas of epidermis of acute GVHD lesions.

In the absence of cutaneous GVHD lesions, monoclonal antibody KL1 revealed discrete alterations in the expression of polypeptides (55–57 kd) in epidermal suprabasal layers, but with no modification of the pemphigus or bullous pemphigoid antigens. In acute GVHD lesions, important alterations were evident in the expression of keratin polypeptides (55–57 kd; Fig. 2) which were sometimes absent in certain regions at all levels of the epidermis; and there was an associated staining (mean intensity) of the basal layer (Figs 3 and 4). Weak and intermittent expression of the bullous pemphigoid antigen was noted, whereas that of the pemphigus antigen was normal.

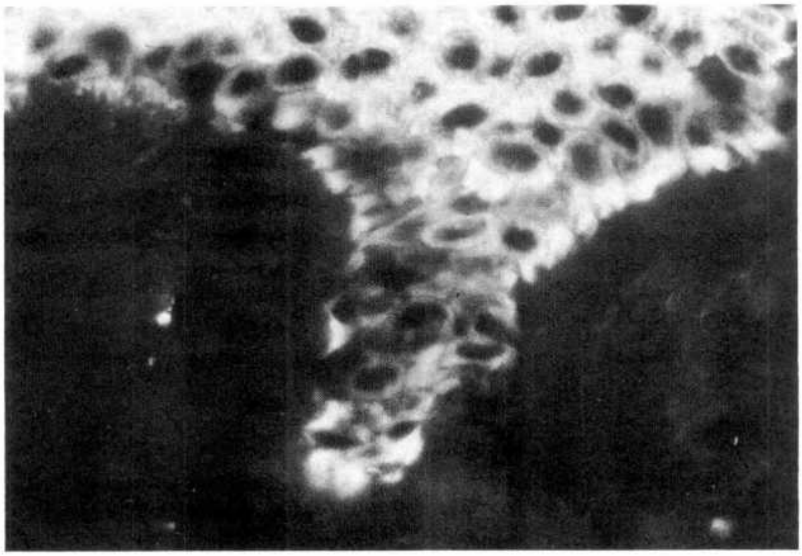

FIGURE 3. Abnormal staining of basal layer in acute GVHD lesions with KL1.

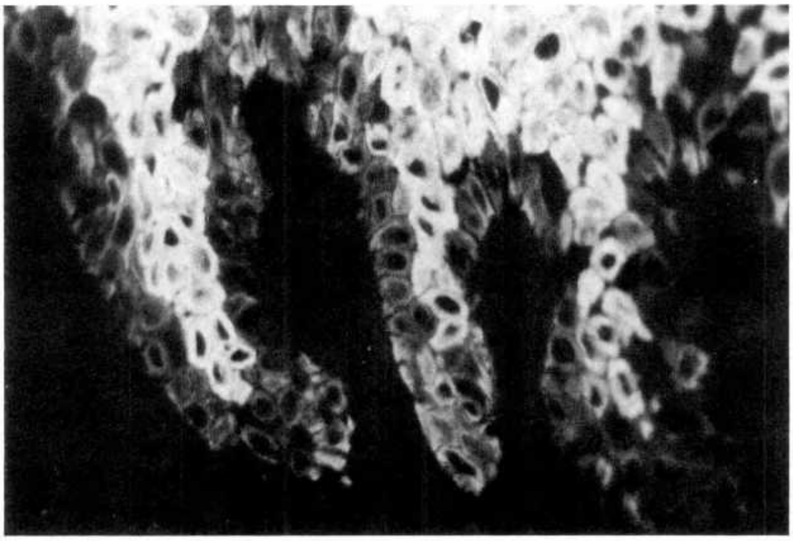

FIGURE 4. Normal control epidermis stained with KL1.

## DISCUSSION

In lesions of acute GVHD, three cells appear to be involved; these are Langerhans cells, lymphocytes and keratinocytes.

For Langerhans cells, our study shows the loss of HLA-DR and IOT6 markers occurring in the absence of cutaneous lesions, which raises the question of the role of chemotherapy and radiotherapy. Regarding Langerhans cells, Belsito *et al.* (1982) and Perreault *et al.* (1984) have both observed a reduction in Langerhans cells in their patients before marrow transplantation. This could be related either to chemotherapy or to the infiltration of bone marrow by tumour cells, affecting the precursors of Langerhans cells.

Subsequently, IOT6 and HLA-DR markers of Langerhans cells reappeared in three of eight patients biopsied on day 100, which is in agreement with the results of both Sloane *et al.* (1984) and of Perreault *et al.* (1984) who indicate an interval of 3–12 months before reappearance of Langerhans cells. However, it is of interest to note that three of the five patients in whom IOT6 and IOT2 staining remained negative at day 100 developed chronic GVHD in the following weeks, which may indicate a possible prognostic value for the interval in reappearance of Langerhans cells in the epidermis. Perreault *et al.* (1984) and Suitters & Lampert (1983) have already suggested a 'prognostic value' for the disappearance of Langerhans cells related to the severity of acute GVHD lesions.

Is it the Langerhans cell markers or the cells themselves which disappear? This question has already been raised concerning ultraviolet irradiation which also causes modifications in Langerhans cell markers (Nordlund, Ackles & Lerner, 1981; Ree, 1982; Aberer *et al.*, 1981). According to Sloane *et al.* (1984), the disappearance of ATPase staining in Langerhans cells observed during GVHD argues in favour of a complete disappearance of Langerhans cells. Recently, Volc-Platzer *et al.* (1984) have proved that Langerhans cells are derived from donor bone marrow precursor cells.

For lymphocytes, our study shows that dermal infiltrate, in the absence of acute GVHD, is scanty (5–10 cells/field); the cells all had HLA-DR receptors and 10% were T-helper lymphocytes. During the course of acute GVHD lesions, two changes were seen. These were cellular polymorphism (40% IOT2+, 40% IOT2+ Leu3+, 20% IOT2+ Leu2+) and intense epidermotropism, by a pure T-suppressor cell population. These results differ from those of Lampert *et al.* (1982) who found a T-suppressor population throughout the dermis and epidermis, but are in agreement with those of Kay *et al.* (1984) and Sloane *et al.* (1984) who studied the T4/T8 ratio during the appearance of cutaneous lesions, finding the ratio higher for lichenoid lesions than for eczematoid lesions. Thus, there is apparently a non-specific ratio of T-helper and T-suppressor cells in the infiltrate of acute GVHD. Chemotherapy and irradiation may play a role in such polymorphism, but this role remains uncertain, especially in the case of patients receiving cyclosporin A.

HLA-DR staining in keratinocytes was found in all six patients with acute GVHD. This is an important diagnostic feature (Suitters & Lampert, 1983; Sloane *et al.*, 1984; Lampert, Suitters & Chisholm, 1981; Mason, Dallman & Barclay, 1981). Biopsies performed at days 15, 30 and 100 (Table 3) show that HLA-DR staining did not precede cutaneous lesions and often disappeared before the clinical changes. The intensity of HLA-DR staining tended to be proportional to the severity of GVHD, a fact already noted by Lampert *et al.* (1982) and Suitters & Lampert (1983) Breathnach & Katz (1983) have shown that the HLA-DR marker is synthesized by keratinocytes and not transmitted from neighbouring Langerhans cells. The significance of this staining, which is seen in other cutaneous diseases (Aiba & Tagami, 1984;

Daynes *et al.*, 1983) is still in question. Breathnach has put forward two hypotheses: that the staining may indicate a state of dedifferentiation of keratinocytes comparable to that of myeloid and erythrocytic cells which are HLA-DR+ at an early stage; or that HLA-DR+ keratinocytes may play the role of antigen presentation, which could account for the HLA-DR+ staining also found in the cells of the intestinal mucosa (Mason *et al.* 1981).

In addition to keratinocyte HLA-DR staining during cutaneous lesions of GVHD, modifications in cytokeratin expression of keratinocytes were noted in our study. This discrete disappearance of the synthesis of the polypeptides in the suprabasal layers of the epidermis in the absence of acute GVHD coincided with treatment, especially radiotherapy. On the other hand, the disappearance of this staining in cutaneous lesions of GVHD was much more widespread throughout the epidermis, clearly affecting the granular layers. These alterations were accompanied by a partial disappearance of the bullous pemphigoid antigen, but with no modification of the pemphigus antigen. Moreover, abnormal synthesis of these polypeptides (55–57 kd) was seen in the basal layer, which is normally not stained (Viac *et al.*, 1983). The significance of this staining by basal cells is unknown.

These observations confirm that keratinocytes undergo important functional alterations during GVHD. However, it is still uncertain whether these alterations are secondary to immunological changes occurring in the epidermis during acute GVHD or whether they are themselves the cause of antigenic modifications (perhaps by means of a virus) in the epidermis.

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# Effects of dicarboxylic acids on normal and malignant melanocytes in culture

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## SUMMARY

We have shown that dicarboxylic acids ($C_9$ and $C_{12}$), known competitive inhibitors of tyrosinase, are selectively cytotoxic to malignant melanogenic melanocytes but not to normal pigmented cells or to amelanotic or non-melanogenic melanoma cells. The main target of this toxicity appears to be the mitochondria, which become markedly swollen and vacuolated. The mechanism of their action has been thought to be due to interference with oxidoreductases in the mitochondria. However, our results suggest that this cytotoxicity most probably does not result simply from inhibition of mitochondrial enzymes, but is closely related to the melanin biosynthesis pathway.

The effects of many agents that have a selective cytotoxicity for pigmented melanoma cells appear to be associated with the activity of tyrosinase in these cells. Dicarboxylic acids, such as azelaic acid (AA) and dodecandioic acid (DA) ($C_9$ and $C_{12}$), known competitive inhibitors of tyrosinase, have been found to be effective in the management of hyperpigmentary disorders, owing to their preferential cytotoxic effects on abnormal melanocytes (Breathnach *et al.*, 1983; Nazzaro-Porro *et al.*, 1979; Nazzaro-Porro *et al.*, 1980; Ward *et al.*, 1984). However, the mechanism of their action is still poorly understood. At first, the depigmenting effects of these diacids were thought to be the result of action on cells with an active tyrosinase system. More recently, the effects have been shown to be the result of action on mitochondrial enzymes (Picardo *et al.*, 1983).

We have examined the effects of AA and DA on melanotic (HFH18-$T^2$) and amelanotic (NP133) sublines of cultured B16 melanoma cells and found that NP133 cells were unaffected by these diacids at concentrations toxic to HFH18-$T^2$ cells (unpublished data). Thus, it appears that their effects are more complex than a mere inhibition of mitochondrial respiration. In this paper we compare the effects of AA and DA on normal and malignant melanocytes and on melanogenic and non-melanogenic cells in culture, in an attempt to explore further the mechanism of their action on pigmented cells.

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## METHODS

### *Cell lines*

Normal choroidal melanocytes were prepared from the eyes of young adult rhesus and cynomolgus macaques (*Macaca mulatta* and *Macaca fascicularis*). The isolation and culture methods have been reported elsewhere (Hu, Pasztor & Teramura, 1977). Cells were grown as short-term cultures, subcultured one to three times and used for experiments within a few weeks.

The melanoma cell lines were sublines of HFH18 designated as HFH18-T$^2$, HFH18–15 and NP 133. The first two lines are melanogenic and the last is non-melanogenic. These three lines were all derived and established from a single B16 mouse melanoma transplanted for many generations in C57BL/6 mice (Hu & Lesney, 1964). These are permanent cell lines, cultured *in vitro* for many years (Hu, 1982).

Another permanent cell line, which was normal and non-melanocytic (non-melanotic and non-melanogenic), was included for comparison. This was derived from the choroid of a 60-day-old normal rhesus foetus (Pasztor & Hu, 1973).

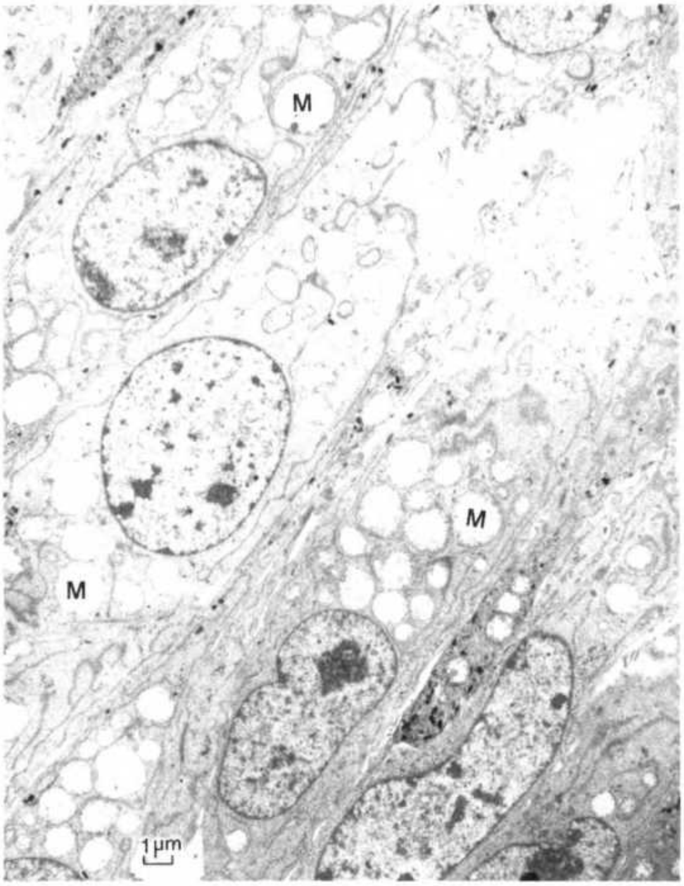

FIGURE 1. HFH18-T$^2$ cells; 9-day culture, DOPA. Azelaic acid 200 $\mu$g/ml (ethanol 1:800), treated for 8 days. Vacuolated mitochondria (M) in cells with or without melanosomes.

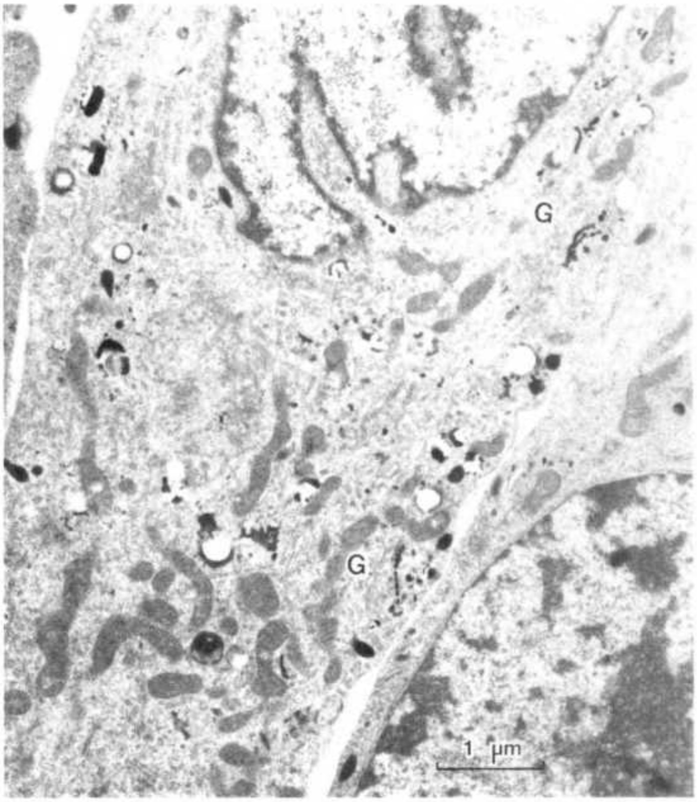

FIGURE 2. HFH18-T$^2$ cells; 10-day culture, DOPA. Ethanol 1:800, treated for 9 days. Note the well-preserved mitochondria and DOPA reaction products in the Golgi (G)-associated vesicles and cisternae.

### *Test agents*

Azelaic acid (AA) and dodecandioic acid (DA) were purchased from the Sigma Chemical Company (St. Louis, MO, U.S.A.). The AA was first dissolved in ethanol (160 mg/ml) and then diluted to 1:800 in Eagle's minimal essential medium with 10% foetal calf serum (MEM-10). The DA was dissolved in ethanol (80 mg/ml) and then was diluted to 1:400 in MEM-10.

### *Treatment of cells*

Cells were treated with AA or DA at concentrations of 100 and 200 μg/ml. Ethanol diluted 1:400 or 1:800 in MEM-10 was used as a control in each experiment. Treatments lasted 3, 6 and 9 days. At the end of each treatment, the cells were fixed and divided into two groups. One group was incubated in 0·1% L-β-3,4-dihydroxyphenylalanine (DOPA) (Sigma Chemical Co.) (White, Hu & Roman, 1983) and the other was incubated in phosphate-buffered saline as a control for the DOPA reaction. The cells were dehydrated and embedded in Epon 812.

The embedded cells were cut into 1 μm sections and stained with toluidine blue for light microscopy. Ultra-thin sections were cut and stained with Reynold's lead citrate and uranyl acetate for electron microscopy.

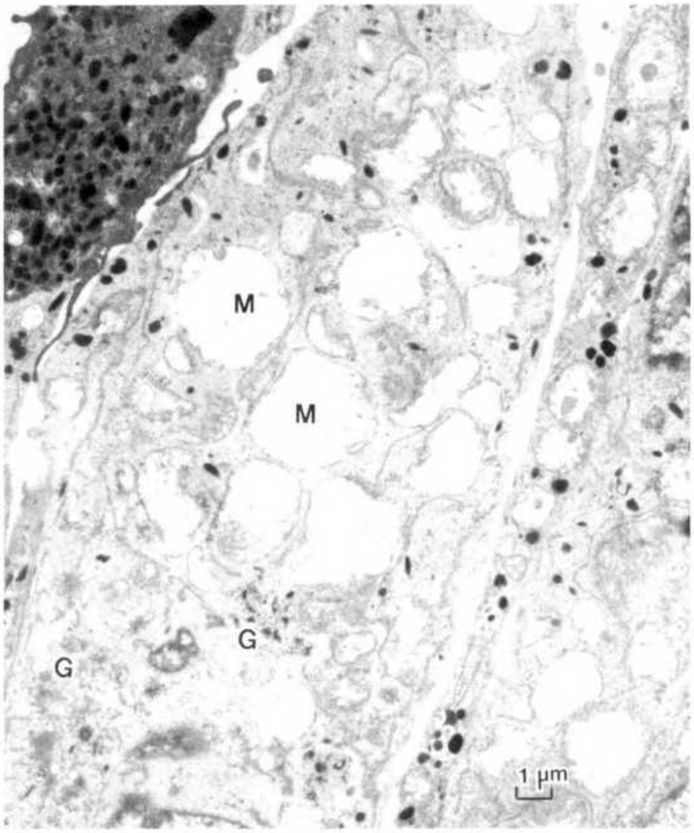

FIGURE 3. HFH18-$T^2$ cells; 10-day culture, DOPA. Dodecandioic acid 200 µg/ml, treated for 9 days. Mitochondrial swelling (M) similar to that seen in Fig. 1. DOPA reaction products present in the Golgi areas (G), but to a lesser extent than in Figure 2.

### *Conditioned medium*

Conditioned medium (CM) was prepared by collection of the culture medium in which the HFH18-$T^2$ cells ($CMT^2$) were growing for 2 or 3 days before confluency. The medium was centrifuged, the sediment was removed and the supernatant was collected and frozen before use. For the CM experiment, the treatments lasted 3 and 6 days only.

In addition, $CMT^2$ was filtered through a 0·45 µm Millipore membrane filter and its effects compared with those of unfiltered $CMT^2$.

## RESULTS

### *Effects of azelaic acid and dodecandioic acid on B16 mouse melanoma cells in culture (HFH18-$T^2$, HFH18-15 and NP133)*

Pigmented melanoma cells after exposure to AA or DA at concentrations of 100 and 200 µg/ml for 3 days had lost much of their pigment and contained vacuoles in the cytoplasm that became more pronounced when the incubation was extended to 5 or 6 days. The culture at first showed

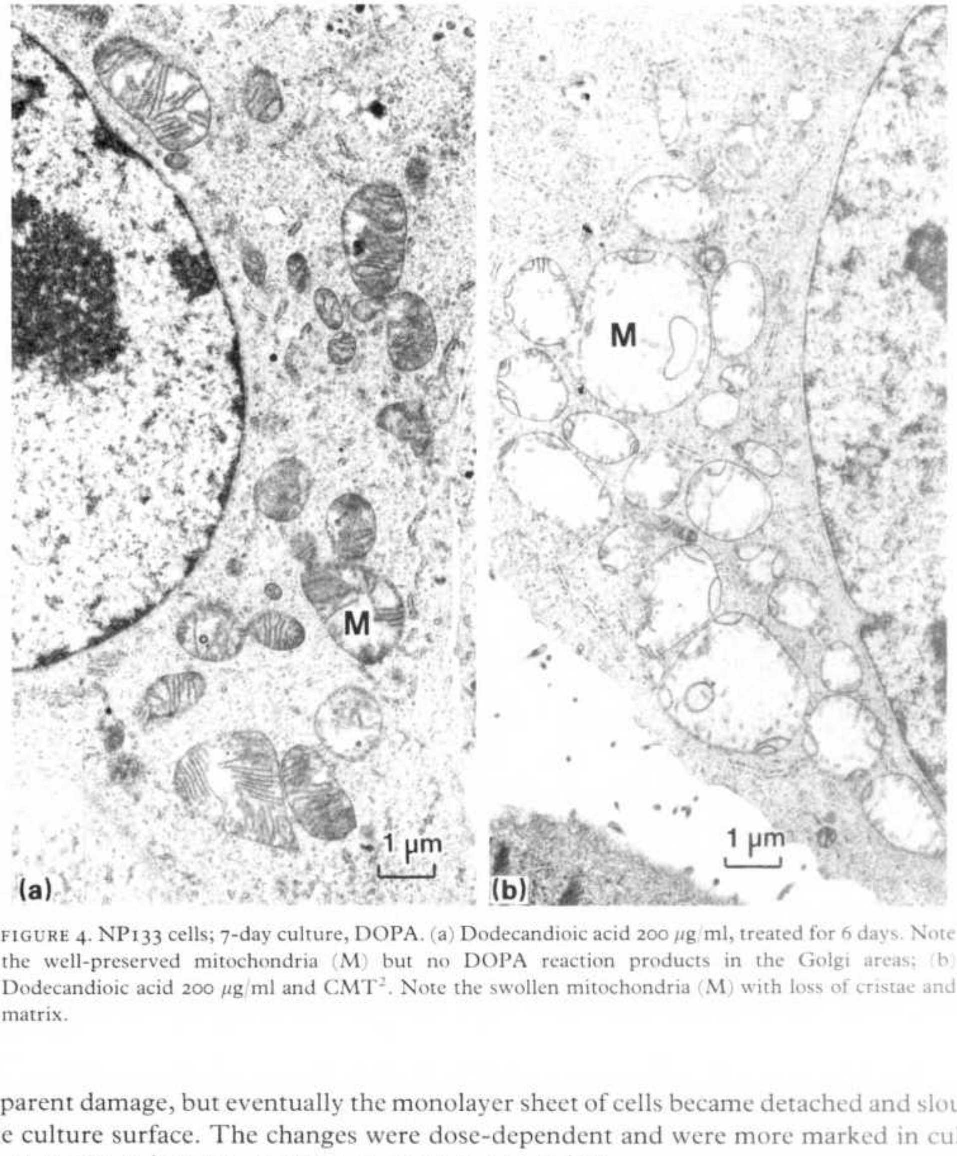

FIGURE 4. NP133 cells; 7-day culture, DOPA. (a) Dodecandioic acid 200 $\mu$g/ml, treated for 6 days. Note the well-preserved mitochondria (M) but no DOPA reaction products in the Golgi areas; (b) Dodecandioic acid 200 $\mu$g/ml and CMT$^2$. Note the swollen mitochondria (M) with loss of cristae and matrix.

no apparent damage, but eventually the monolayer sheet of cells became detached and sloughed off the culture surface. The changes were dose-dependent and were more marked in cultures exposed to 200 $\mu$g/ml than in those exposed to 100 $\mu$g/ml.

Ultrastructurally, the vacuoles were markedly swollen mitochondria many times larger than normal. The mitochondria appeared vacuolated because they had lost most of the cristae and matrix. However, the mitochondrial external envelope was preserved. Other organelles in these cells appeared intact except for the Golgi-associated vesicles and cisternae, which either contained reduced amounts of, or completely lacked, DOPA reaction products. These cells contrasted with the untreated control cells, which normally showed extensive deposits of DOPA reaction products in the Golgi-associated endoplasmic reticulum (RER) and lysosomes.

However, the NP133 cells, the non-pigmented and non-melanogenic melanoma line, derived from the same B16 tumour as the HFH18-T$^2$ cells were not affected by the diacids and showed no mitochondrial swelling at AA or DA doses of 100 or 200 $\mu$g/ml. When these cells were

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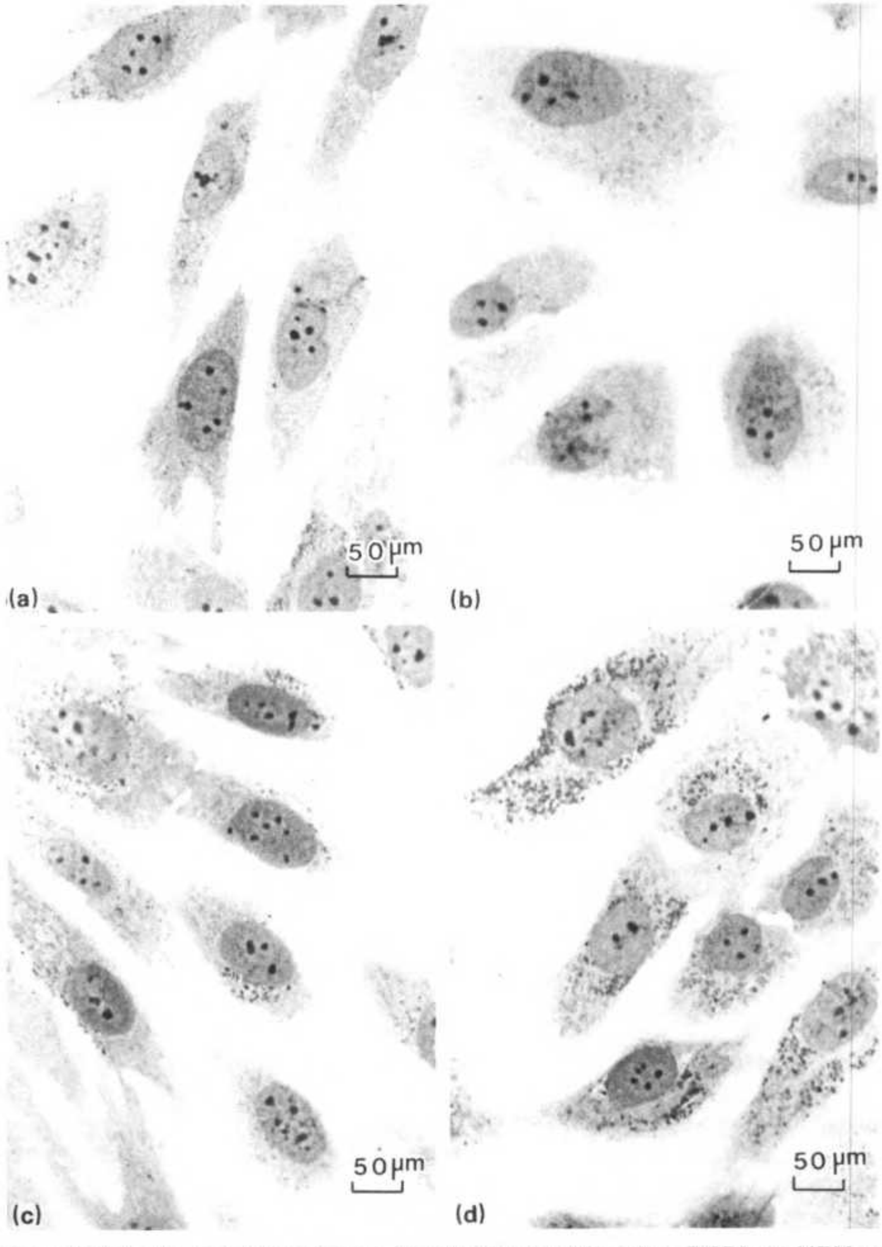

FIGURE 5. RF/6A cells, monolayer culture: 7-day culture; treated for 6 days, DOPA; (a) MEM-10; (b) Dodecandioic acid (DA) 200 $\mu$g/ml; (c) CMT$^2$ and ethanol (1:400). Note there are more granules than in cells treated with DA (b), but less than in cells treated with DA plus CMT$^2$ (d); (d) Dodecandioic acid 200 $\mu$g/ml and CMT$^2$. Note the many darkly stained granules in the cytoplasm. These cells have more granules than do cells in (a), (b) or (c).

incubated in a medium containing $CMT^2$ their responses to these diacids were similar to the responses of HFH18-$T^2$ cells.

*Effects of azelaic acid and dodecandioic acid on uveal melanocytes of adult rhesus and cynomolgus macaques*

At the light microscopic level, neither iridial nor choroidal melanocytes of adult rhesus and cynomolgus macaques showed apparent damage from AA or DA at concentrations toxic to HFH18 cells. Ultrastructurally, the treated cells showed fusion of melanosomes and some loss of melanosome ultrastructures, but the mitochondria were well preserved. There was no detectable change in tyrosinase activity, as shown by the amount of DOPA reaction products. Instead, in the Golgi-associated vesicles and cisternae of treated cells there may even have been some increase in the DOPA reaction products.

*Effects of dodecandioic acid on non-pigmented and non-melanocytic rhesus cells (RF/6A)*

The addition of 200 μg/ml of DA to cultures of RF/6A cells had no noticeable effect, but when $CMT^2$ was added with DA, the cytoplasm of these cells showed many dark granules of varying sizes and shapes. The RF/6A cells, when grown in control media (MEM-10 and MEM-10 plus ethanol 1:400) or in medium containing DA at 200 μg/ml, contained some of these granules, but they were smaller, fewer and not as dark. When cells were grown in media containing $CMT^2$ and $CMT^2$ plus ethanol, there were more of these granules. Cells grown in $CMT^2$ plus 200 μg/ml of DA had the most granules.

Under the electron microscope, these granules were irregularly electron-dense. Some were round and many had irregularities in shape and varied in size. They had an amorphous or granular background and often contained myelin figures. These granules appeared to be secondary lysosomes.

As in the case of NP133 cells, but to a lesser extent, $CMT^2$ plus DA induced mitochondrial swelling with partial loss of the cristae and granular matrix. This effect was not seen in RF/6A cells grown in medium with $CMT^2$ or DA alone.

There were no DOPA reaction products in the Golgi-associated vesicles and cisternae in any of these cells. In RF/6A cells cultured in $CMT^2$, the lysosomal granules most probably were derived from the $T^2$ cells, and they could have been degraded melanosomes phagocytosed by the RF/6A cells. Swelling of the mitochondria was caused probably by the action of DA combined with the intermediate products in tyrosine-melanin metabolism of HFH18-$T^2$ cells.

In order to determine whether these lysosomal granules were actually degraded melanosomes that were phagocytosed by RF/6A cells in culture, the filtered $CMT^2$ was used instead of the unfiltered medium, which contained some particulate material such as extracellular melanosomes. There were no striking differences in the amounts of granules in cells grown in the unfiltered medium containing DA at 200 μg/ml, but the lysosomal granules in the cells grown in unfiltered medium contained many melanosomes in addition to the amorphous materials and myelin figures characteristic of cells in the filtered medium. Ultrastructurally, both filtered and unfiltered $CMT^2$ in combination with DA at 200 μg/ml induced swelling and vacuolization of mitochondria. Therefore, it appears that whatever causes swelling and vacuolization of mitochondria is not directly related to the particulate melanosomes, but to the more soluble components of $CMT^2$.

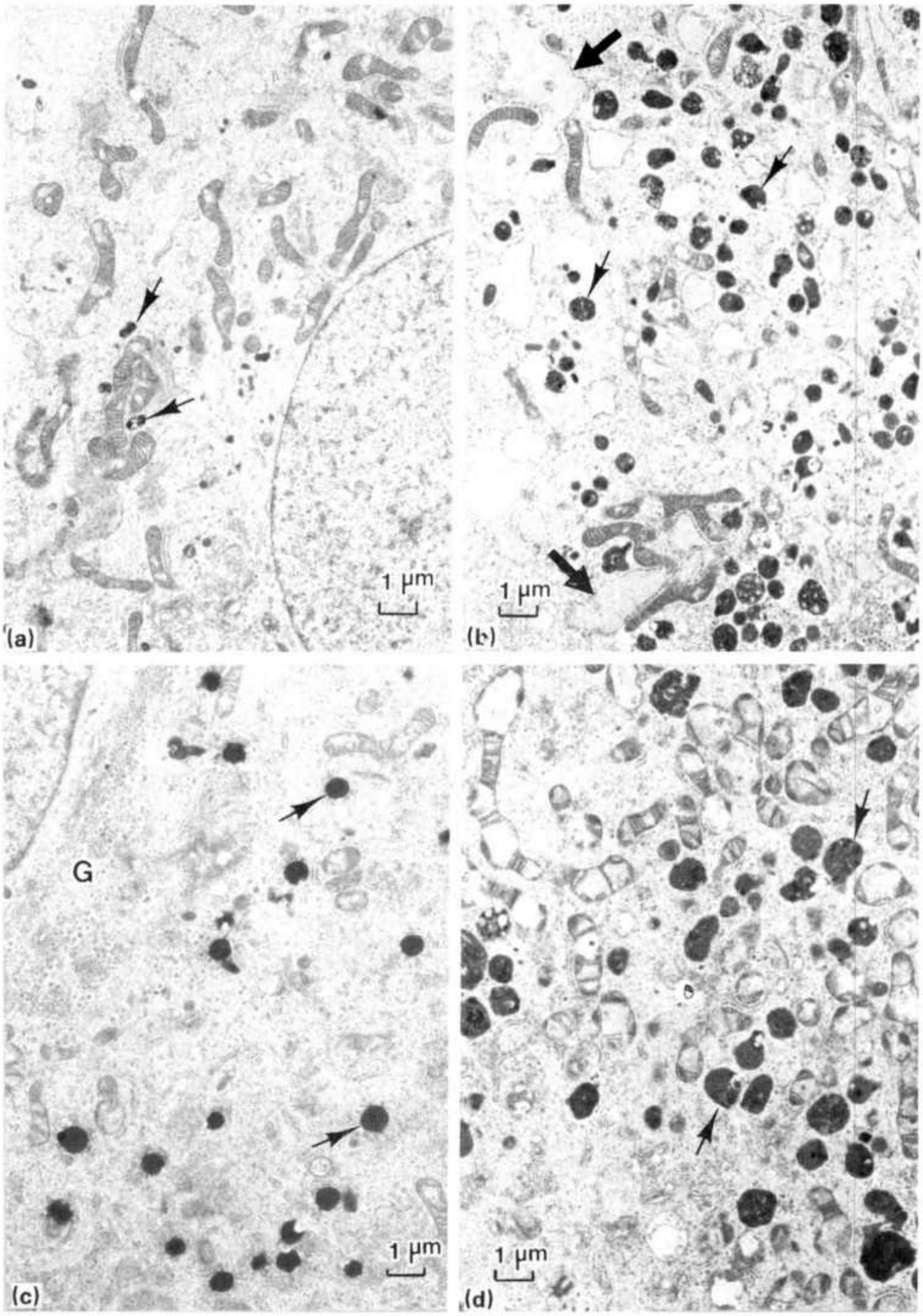

FIGURE 6. RF/6A cells; monolayer: 7-day culture; treated for 6 days, DOPA; (a) MEM-10. The mitochondria are well preserved. Note the few scattered, dense granules (arrows); (b) CMT$^2$ and ethanol (1:400). The mitochondria are still well preserved. There are many more dense bodies (arrows) than in cells cultured in MEM-10 (a). The RER (thick arrows) is dilated and filled with some flocculent

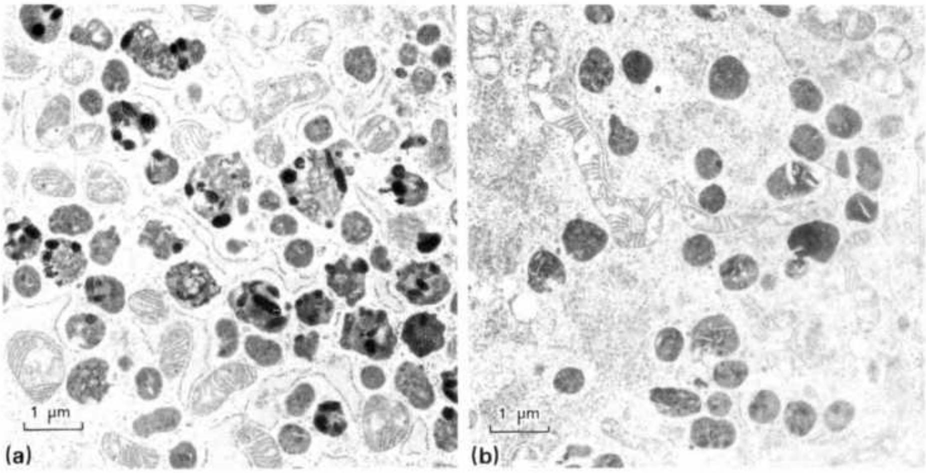

FIGURE 7. RF/6A cells; 7-day culture, 6-day treatment; (a) DA 200 $\mu$g/ml and CMT$^2$. Note the many large secondary lysosomes containing amorphous materials and melanosomes. The mitochondria are large and vacuolated, with some loss of cristae. The Golgi resemble those in 6(a). The RER is seen predominantly as small segments. Note also the large amount of greatly distended RER filled with amorphous material; (b) DA 200 $\mu$g/ml and filtered CMT$^2$. Note the many secondary lysosomes which are not as large as those in (a) and contain amorphous materials, myelin figures and no melanosomes. The mitochondria show changes similar to those in (a); the Golgi areas are not remarkable. The RER shows some dilatation, but it is not as extensive as that seen in (a).

## DISCUSSION

In a recent paper, Ward *et al.* (1984), on the basis of their analytical, ultrastructural, autoradiographic and biochemical studies, suggested that the toxicity of dicarboxylic acids in melanoma cells is not related to anti-tyrosinase activity but may be due to interference with oxidoreductases (NADH dehydrogenase, succinic dehydrogenase and reduced ubiquinone cytochrome c oxidoreductase). However, these observations do not explain why our melanotic cells were more sensitive to the cytotoxic effects of the diacids than were the amelanotic cells. That cytotoxicity was enhanced in our non-melanotic and non-melanogenic NP and RF/6A cells when CMT$^2$ was added to the culture system suggests the involvement of active tyrosinase, metabolites of tyrosine-melanin synthesis, or both in this cytotoxic reaction.

Uveal melanocytes (Hu & Mah, 1979; 1983), especially choroidal melanocytes of the eyes of adult macaques, are classified as terminally differentiated cells; they do not undergo active division and only rarely incorporate thymidine (unpublished data). Pigmented choroidal melanocytes have mature, highly melanized melanosomes but have only low levels of tyrosinase and gamma-glutamyl transpeptidase activity. Ultrastructurally, they are replete with melanosomes but contain only small numbers of mitochondria, ribosomes, endoplasmic reticulum, small and underdeveloped Golgi complexes and other organelles (Hu & Mah, 1983). Although

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material. Ethanol treatment resulted in similar changes, but there was no increase in dense bodies in the cytoplasm. (c) DA 200 $\mu$g/ml. The changes are similar to those seen in (b), except that there is no increase in dense bodies; (d) DA 200 $\mu$g/ml and CMT$^2$. Note the mitochondria are larger and appear partially vacuolated with some loss of cristae and matrix. There are many dense bodies, more and larger than those seen in (a), (b) or (c). The dense bodies appear to be secondary lysosomes; they are irregular in shape, of uneven density and contain myelin-like figures and some flocculent materials.

there are DOPA reaction products in the Golgi-associated vesicles and cisternae, the amounts are small (Hu & Mah, 1979; 1983).

These findings constitute a profile of a metabolically inactive cell-type. Perhaps the response to the cytotoxic effects of the two diacids is related to active metabolism and enzyme activities of the cells involved. Thus, the inactive cells are less affected by the toxicity. That both NP133 and RF/6A cells are metabolically as active as HFH18-$T^2$ cells, except in terms of melanogenesis, rules out active cell metabolism as the only prerequisite and suggests the participation of the melanogenic pathway in this cytotoxicity.

Demopoulos, Gerving and Bagdoyan (1965) observed a segment of respiration depressed by tyrosinase inhibitors, such as phenyl lactate, in human and murine melanomas. The concomitant rise in aerobic glycolysis that occurred when this segment of respiration was blocked suggested that the inhibited portion of respiration was vital and was possibly involved in oxidative phosphorylation. Amelanotic melanoma cells do not possess a segment of respiration susceptible to tyrosinase inhibitors. Whether this peculiarity contributes to the differential toxicities of AA and DA in melanogenic and non-melanogenic cells is not yet known.

## ACKNOWLEDGMENTS

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# The histopathology of localized lipoatrophy

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## SUMMARY

We studied 11 patients with the clinical diagnosis of lipoatrophy and found two histopathological subsets. Six patients presented with a distinctive picture, which we termed 'involutional' fat, consisting of lobules of small lipocytes embedded in hyaline connective tissue with numerous capillaries. Five of these six patients had a single lesion, usually of the upper arm. Serological studies were normal, and direct immunofluorescence, performed in three cases, showed immunoreactants in the blood vessels in only one. The four patients with inflammation of the fat had multiple areas of localized lipoatrophy. Three had biopsies for direct immunofluorescence and all three showed immunoreactants involving the basement membrane zone (two cases) or blood vessels (one case); and three had serological abnormalities. We suggest that the involutional histopathological pattern is a distinctive subset of localized lipoatrophy.

Localized absence of the panniculus generally results in the clinical presentation of well-defined depressions of the skin. Although this condition is easily observed, the clinical importance and histopathological features of such lesions usually have been difficult to interpret. Traditionally, this disorder has been designated idiopathic localized lipoatrophy and the histopathology has been considered to be normal or to show only diminished or absent adipose tissue.

We have studied 11 patients with so-called idiopathic localized lipoatrophy and found that 10 of them had definite abnormalities on deep excisional biopsy.

## CASES

We studied 11 patients with the clinical diagnosis of lipoatrophy, who underwent deep excision biopsies that included subcutaneous tissue. Of the 11 patients, 10 were female, ranging in age from 14 to 56 years, and one was a 5-year-old boy. The boy had the unique findings of regrowth of fat and associated vitiligo on follow-up.

The patients presented from 2 months to 7 years after onset of the lesions. Five patients (cases 2–6) presented with an isolated area of localized atrophy and the other patients had two or more lesions. The lesions in all patients tended to be proximally located; the buttocks, thighs and

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TABLE 1. Grouping of 11 patients with lipoatrophy according to histopathology

| Histopathological group | Case no. | Age (years) | Sex | Lesion(s) Location | Lesion(s) Duration* |
|---|---|---|---|---|---|
| Involutional | | | | | |
| | 1 | 41 | F | L hip | 6 months |
| | 2 | 5 | M | R upper arm† | 2 months |
| | 3 | 14 | F | L upper arm† | 1 year |
| | 4 | 31 | F | L upper arm† | 1 year |
| | 5 | 28 | F | L hip† | 6 months |
| | 6 | 20 | F | R upper arm† | 1 year |
| Inflammatory | | | | | |
| | 7 | 34 | F | R and L upper arms | 4 years |
| | 8 | 56 | F | R and L lower legs | 1 year |
| | 9 | 49 | F | Arms, thighs, buttocks | 1–2 years |
| | 10 | 32 | F | R arm, L arm, hips, thighs | 7 years |
| Normal | | | | | |
| | 11 | 26 | F | L upper arm, thigh, R and L lower legs | 2 months |

* Duration of lesion(s) prior to presentation.

† Only one lesion present.

upper arms were involved more frequently than the lower legs. The face, scalp, breasts, distal aspects of the extremities and mucous membranes were not affected (Table 1).

Most of the patients had a localized depression, up to 6 × 8 cm, without overlying cutaneous changes (Fig. 1). Case 9 had depressed lesions with purpura of the overlying skin. Two patients (cases 5 and 7) had a history of deep injections 10 years or more prior to the onset of the atrophy. In case 7, the lesions began after trauma to the arms from beating; bruising was noted initially and then atrophy. Although trauma may have played a role in the onset of these lesions, the histopathological findings did not indicate traumatic or factitial panniculitis.

The patients generally had no underlying diseases or associated symptoms at the time of presentation, although three patients reported arthralgias. One patient (case 1) had had recurrent rheumatic fever during childhood and had a maternal aunt with systemic lupus erythematosus.

## LABORATORY INVESTIGATIONS

### *Serology*

Serological studies were performed in 10 of the 11 patients and were negative or normal in all but three (Table 2). Of six patients tested for extractable nuclear antigen (ENA), one (case 10) had positive results. This case was ribonucleoprotein positive, Smith antigen negative and Sjögren's syndrome B antigen negative, and was also the only case positive for anti-nuclear antibody (titre

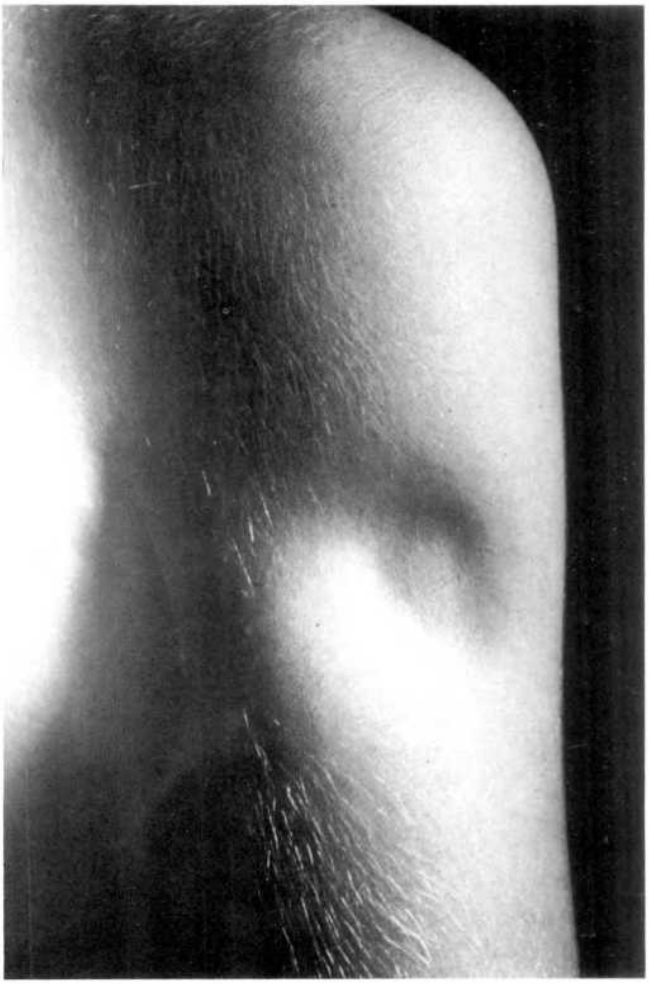

FIGURE 1. Typical clinical presentation of lipoatrophy. Localized depression in lateral aspect of upper arm.

1:64) out of 10 patients tested. Complement levels were determined in six patients. One patient (case 8) had a slight decrease in C3 (86 mg/dl; normal, 88–206), but had normal levels of CH50 and C4. One patient (case 7) showed a slight increase in anti-n-DNA (0·95 μg/ml; normal, 0–0·80 μg/ml) out of seven patients tested.

### *Direct immunofluorescence*

Direct immunofluorescence was performed on freshly frozen tissue. Fluorescein-conjugated antisera (IgG, IgM, IgA, C3 and fibrinogen) were diluted with veronal buffer, pH 7·5, to a suitable concentration as determined by Ouchterlony diffusion. Biopsy specimens for direct immunofluorescence were obtained from seven patients and showed deposition of immunoreactants in six. Two cases had granular deposition of IgM or IgM plus C3 at the basement membrane zone, three showed staining of dermal vessels and one showed only dermal-epidermal cytoid bodies with IgM. One case gave entirely negative results.

TABLE 2. Results of direct immunofluorescence and serological tests in 11 patients with lipoatrophy

| Histopathological group, | Case no. | Direct immunofluorescence | Serological tests* | |
|---|---|---|---|---|
| | | | Normal | Abnormal |
| Involutional | | | | |
| | 1 | Cytoid bodies with IgM | ENA, ANA anti-n-DNA | |
| | 2 | BV, IgM, IgA, fibrinogen | ANA, anti-n-DNA | |
| | 3 | ND | ND | |
| | 4 | ND | ANA | |
| | 5 | Negative | ANA, complement | |
| | 6 | ND | ANA | |
| Inflammatory | | | | |
| | 7 | BM IgM complement | ANA, ENA, anti-n-DNA | Slight increase anti-n-DNA |
| | 8 | BV fibrinogen | ANA, ENA, anti-n-DNA | Slight decrease C3 |
| | 9 | BM, IgM, C3 | ANA, ENA, anti-n-DNA, complement | |
| | 10 | ND | Anti-n-DNA complement | ANA+ ENA+ |
| Normal | | | | |
| | 11 | BV, IgM, C3, fibrinogen | ANA, anti-n-DNA, ENA, complement | |

*ANA, anti-nuclear antibody; BM, basement membrane; BV, blood vessel; ENA, extractable nuclear antigen; +, positive; ND, not done.

Cutaneous leukocyte monoclonal antibody studies were performed on two freshly frozen biopsy specimens from the panniculus (from cases 8 and 9) according to a standard peroxidase-antiperoxidase technique using six antisera: Leu-1, Leu-2a, Leu-3a (Becton-Dickenson), OKT6, OKM1 (Ortho) and B1 (Coulter), followed by incubation in goat anti-mouse IgG (peroxidase-conjugated). One specimen (from case 9) showed positive staining of approximately 30% of the cells with Leu-1 (pan T-cell), less than 10% with Leu-2a, 30% with Leu-3a, less than 10% with OKM1 (monocytes) and no staining with B1. The other specimen (from case 8) showed focal perivascular staining of mononuclear cells with Leu-1 and no staining with Leu-2a or B1. Approximately 10% of cells stained with Leu-3a, either scattered or in clusters in the panniculus, and 40% stained positively with OKM1, in clusters in the panniculus.

### *Histopathology*

Multiple biopsy sections stained with haematoxylin and eosin were examined from all 11 patients. In addition, special stains, including alcian blue-PAS, Hale's colloidal iron, reticulin, von Kossa, Masson trichrome and aldehyde-fuchsin-elastin-Giemsa, were used.

The histological findings fell into two distinct groups: inflammatory and involutional (Table 3).

TABLE 3. Histopathology of 11 patients with lipoatrophy

| Histopathological group | Features |
|---|---|
| Involutional | Lobules of small- to medium-sized lipocytes on background of hyaline material<br>Peripheral lobular accentuation<br>Absence or scarcity of inflammatory cells<br>Myxoid stroma<br>Numerous capillaries |
| Inflammatory | Normal-appearing lipocytes<br>Normal vasculature<br>Scattered focal lymphocytes, histiocytes and plasma cells |

Cases 7 to 10 had inflammation within the fat. Cases 8 and 9 had mild lymphocytic inflammation in a perivascular location and in focal periseptal regions of fat lobules; they also had minimal septal fibrosis. Case 10 showed focal fibrosis and hyaline degeneration in the fat as well as lymphocytic inflammation. The biopsy specimen from case 7 showed more inflammation than the others and had lymphoplasmacytic aggregates on a background of hyaline degeneration in broad areas of the fat, a finding reminiscent of lupus panniculitis. This specimen showed focal haemorrhage and haemosiderin-laden macrophages. No involutional fat changes (as described below) were observed in these four patients.

The findings in the involutional group (cases 1–6) were different and striking. Adipose tissue was present but was abnormal. The biopsy specimens showed well-defined, various-sized lobules of fat with small- to medium-sized lipocytes on a background of hyaline material (Fig. 2). Individual lipocytes showed substantial variation in size but were generally smaller than normal and often appeared to be rounder and to have more prominent nuclei than normal fat cells. At low power, the lobules sometimes appeared eosinophilic because of the abundant

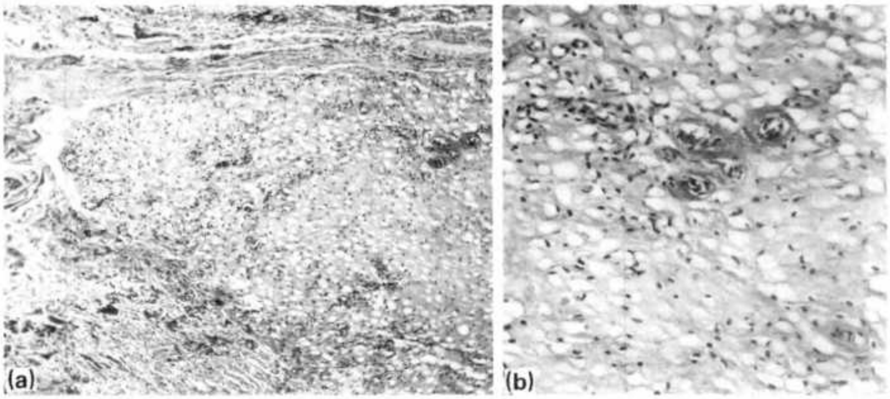

FIGURE 2. Involutional pattern of lipoatrophy. (a) Well-defined fat lobule composed of numerous small lipocytes with intervening hyaline tissue (involutional lipoatrophy). (Haematoxylin and eosin; original × 50). (b) Higher-power view of fat lobule shows prominent capillaries within the lesion and small lipocytes on a background of hyaline stroma. (Haematoxylin and eosin; original × 125).

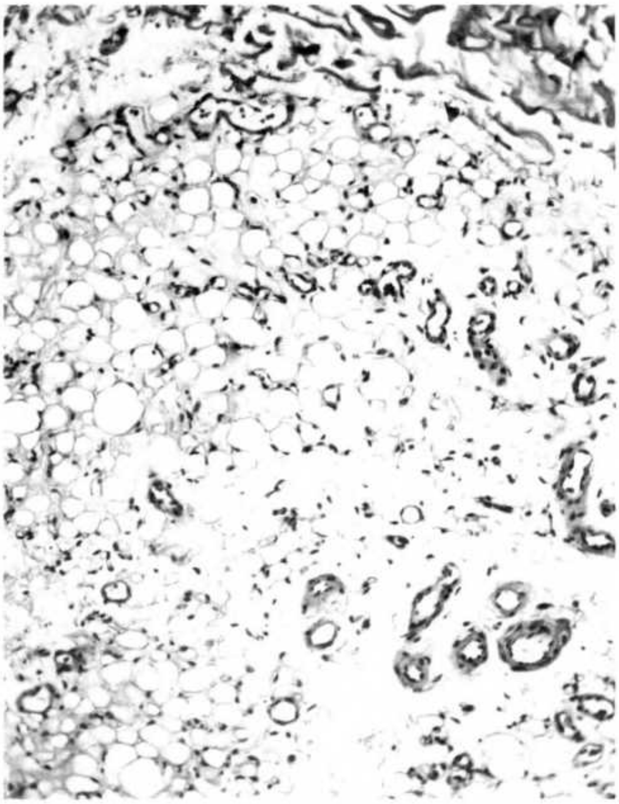

FIGURE 3. Involutional pattern of lipoatrophy. Small, various-sized lipocytes but less intervening stroma than in Fig. 2. Numerous capillaries are evident. (Haematoxylin and eosin; original × 138).

hyaline material intervening between individual lipocytes. The quantity of this material in the biopsy specimens varied from sparse (Fig. 3) to copious (Fig. 2); with abundant material, the small lipocytes seemed to be embedded in a hyaline sea. In addition, capillaries appeared abnormally numerous and prominent. These lobules exhibited a pattern reminiscent of foetal fat. In two patients, biopsy specimens showed areas of normal fat in addition to the involutional tissue. In these cases, the involutional change was evident at the periphery of the lobule and the fat appeared more normal towards the centre. Several specimens had lobules showing myxoid degeneration; these lobules were composed of a few very small lipocytes embedded in an oedematous background with numerous capillaries and focal lipolysis (Fig. 4).

Special staining for calcium was negative and reticulin was absent except around capillaries. Aldehyde-fuchsin-elastin-Giemsa staining showed scattered, fine, curled elastic fibres within the lobules in the intervening hyaline zone in the cases with a well-developed involutional pattern (Fig. 5). Trichrome stain revealed weak homogeneous staining in these areas, much less intense than that surrounding capillaries or that of the dermal collagen. Scattered small foci of granular mucin-positive cells were present in four cases (Fig. 6).

Although occasional individual round cells, consistent with mononuclear inflammatory cells, were seen, this change, when present, was minimal in contrast to the infiltrate in the inflammatory group.

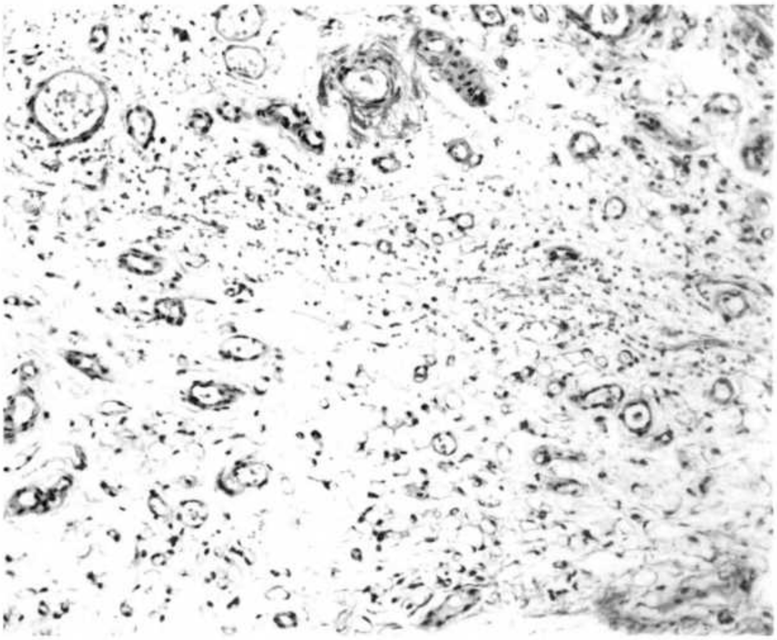

FIGURE 4. Involutional pattern of lipoatrophy. Myxoid lobule containing numerous capillaries and a few lipocytes on an oedematous background with focal lipolysis. (Haematoxylin and eosin; original × 136).

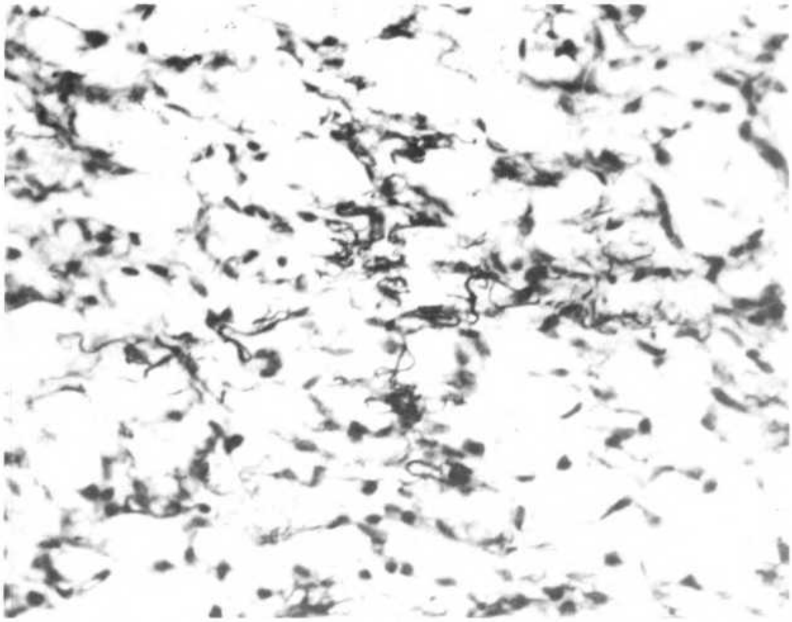

FIGURE 5. Involutional pattern of lipoatrophy. Elastin stain shows fine, curled elastic fibres in the hyaline zone between lipocytes. (Elastin-Giemsa; original × 360).

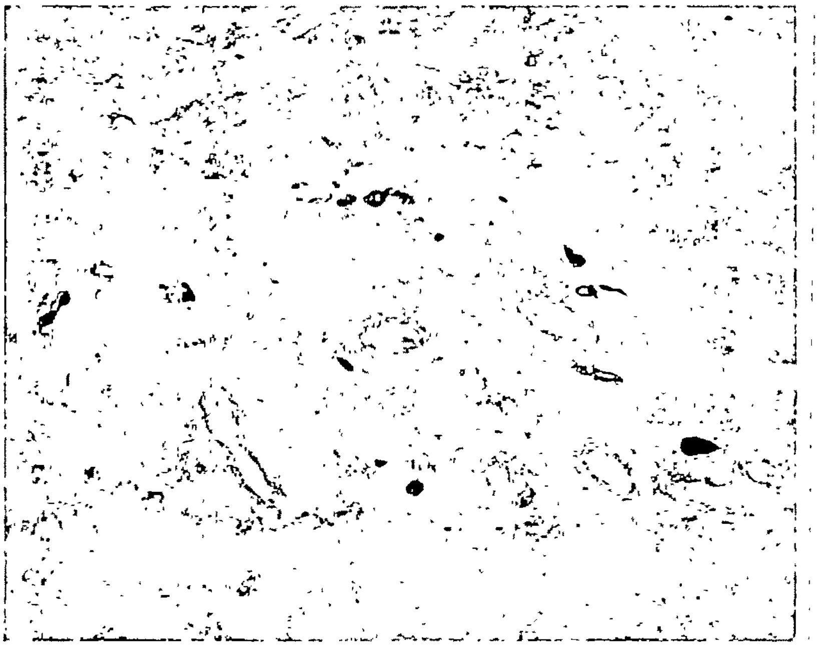

FIGURE 6. Involutional pattern of lipoatrophy. Focal mucin-positive cells are seen in a perivascular location. (Hale's colloidal iron; original × 225).

One patient (case 11) showed no significant subcutaneous histopathological changes, but immunofluorescence showed reactants in the blood vessels.

## DISCUSSION

Traditionally, histological diagnosis of a subcutaneous nodule has been much easier than that of an area of subcutaneous atrophy, and the histopathological features of localized lipoatrophy have often been considered insignificant. Previous reports have noted various degrees of inflammation in localized lipoatrophy (Bettley, 1950; Gschwandtner & Münzberger, 1974; Jablonska, Szczepanski & Gorkiewicz, 1975; Imamura *et al.*, 1979; Lee *et al.*, 1982). Gschwandtner & Münzberger (1975) emphasized the presence of giant cells within the panniculus in some cases—perhaps an indication of another type of inflammation that was not observed in our patients. Reed, Clark and Mihm (1973) described the involutional pattern in a report on diseases of the fat, but they did not include specific case histories. Bruinsma (1967) referred to 'atrophic fat cells' and 'sclerotizing' of subcutaneous tissue. Gschwandtner and Munzberger (1975) noted prominent capillaries with endothelial swelling and small lipocytes, but photomicrographs of biopsy specimens from their patients did not show the involutional pattern. We have previously described four patients with localized lipoatrophy, all of whom had inflammation within the panniculus (Peters & Winkelmann, 1980). Our present group of patients, in which 10 out of 11 were found to have abnormalities on deep excisional biopsy, underscores the value of histopathological investigation in patients with idiopathic localized loss of fat.

The findings in our patients show a distinctive histopathological pattern that we have termed 'involutional fat' because the lobules with small lipocytes and numerous capillaries resemble immature adipose tissue. The clinical and laboratory findings in our patients suggest that the involutional and inflammatory histological subsets may indeed reflect two different clinical patterns of disease. Of the six patients with the involutional type of histopathology, five had only a single, isolated area of lipoatrophy. A unique finding in one of these patients was complete resolution of the lesion. This patient had an area of lipoatrophy (3 cm × 3 cm) over the upper part of the right arm of 2 months' duration and 4 months later had re-formation of the fat. In contrast, all four patients with the inflammatory pattern presented with multiple areas of localized lipoatrophy. Only three of our 11 patients had serological abnormalities; one showed a slight decrease in C3, one a slight increase in anti-n-DNA, and one was positive for anti-nuclear antibody and ENA. Although these changes alone are not diagnostic of a particular connective tissue disease, it seems important that all three of these patients had the inflammatory rather than the involutional histological pattern. Similarly, the two patients with immunoreactants at the basement membrane zone (IgM in a granular pattern with or without C3) also were in the inflammatory group, while the patients with the involutional pattern showed non-specific dermal vessel fluorescence, cytoids, or no immunoreactants.

The recognition of distinct histopathological subsets of lipoatrophy may have diagnostic and therapeutic implications. Localized lipoatrophy may be the only presenting feature in patients with underlying or developing connective tissue disease, particularly lupus erythematosus, scleroderma or morphea (Person & Su, 1979; Winkelmann & Peters, 1982; Winkelmann, 1983). In these situations, the histological pattern may be the only means of diagnosis if laboratory data or other clinical features are unremarkable. Reports of patients with lipoatrophy or lipodystrophy and associated immunological abnormalities have also provided evidence that many cases of lipoatrophy are a manifestation of immunological disease (Ziff, 1974; Alarcón-Segovia & Ramos-Niembro, 1976; Ipp *et al.*, 1976; Sissons *et al.*, 1976; Hall, Gillespie & Tenczynski, 1978; Wilson, Sissons & Morgan, 1978). Patients with partial or localized lipoatrophy have shown hypocomplementaemia, thyroiditis, systemic lupus erythematosus, Sjögren's syndrome, idiopathic thrombocytopenic purpura and glomerulonephritis. Recently, Brickman *et al.* (1984) described a patient with partial lipodystrophy and hereditary angioedema—a finding again demonstrating that such patients should be thoroughly investigated.

The microscopic features in the six biopsy specimens showing involutional lipoatrophy are dramatically different from those from patients with inflammatory lipoatrophy. It would be informative if the involutional histological picture, in contrast to the inflammatory pattern, could be correlated with an absence of underlying or associated diseases. However, one patient with the involutional pattern had arthralgias, a history of recurrent rheumatic fever and a maternal aunt with systemic lupus erythematosus. In addition, because of the development of vitiligo in another patient with a positive family history of vitiligo, we are cautious about making absolute clinicopathological distinctions between the two groups at this time. Although the possibility that the two patterns represent different phases of the same pathological process cannot be ruled out, no case of inflammatory lipoatrophy has subsequently or coincidentally shown the involutional pattern, including our four previously reported cases (Peters & Winkelmann, 1980). Limited clinical involvement, as well as the absence of serological changes or important direct immunofluorescence findings in the patients with the involutional pattern, suggests a form of disease that may be more benign than that in patients with the inflammatory features.

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# Some effects of calcitonin gene-related peptide in human skin and on histamine release

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## SUMMARY

Calcitonin gene-related peptide (CGRP) produced a dose-related wheal and flare reaction in human skin at doses of 12·5 to 50 pmol. The flare response but not the wheal response to CGRP and substance P were inhibited by prior treatment of the subject with oral chlorpheniramine, 16 mg. CGRP, but not substance P, was potent in producing a delayed erythema and surrounding pallor in human skin, which peaked at 1 h and persisted for more than 3 h after injection, when wheal and flare responses had subsided. The delayed response was accompanied by infiltration of polymorphonuclear leukocytes. The delayed erythema and pallor produced in response to CGRP were not inhibited by oral chlorpheniramine, or by 4% prilocaine injected locally. CGRP released histamine from rat peritoneal mast cells over the concentration range 2·5–10 $\mu$M. CGRP was about fourfold less potent than substance P in releasing histamine. The substance P analogue, [D-Pro$^4$, D-Trp$^{7,9,10}$]SP$_{4-11}$ 10 $\mu$M, and benzalkonium chloride 10 $\mu$M inhibited histamine release from rat mast cells stimulated by either CGRP or substance P.

Calcitonin gene-related peptide (CGRP) contains 37 amino acid residues and its occurrence was predicted on the basis of alternative processing of RNA transcribed from the calcitonin gene (Rosenfeld *et al.*, 1983). CGRP is produced in neural tissue by an mRNA that is distinct from that in thyroidal C cells where calcitonin is normally generated. CGRP has a wide distribution in neuronal tissue and also in glandular tissues (Rosenfeld *et al.*, 1983; Tschopp, Tobler & Fischer, 1984; Fischer *et al.*, 1985). The novel peptide has been shown to have a number of biological activities including contraction of the guinea-pig ileum (Tippins *et al.*, 1984), stimulation of sympathetic outflow (Fisher *et al.*, 1983) and vasodilation (Brain *et al.*, 1985). CGRP and substance P-like immunoreactivity have been shown to co-exist in dorsal root ganglion cells and the spinal cord (Terenghi *et al.*, 1985; Fischer *et al.*, 1985). CGRP is present in peripheral as well as the central terminals of sensory neurones, as is substance P (Rosenfeld *et al.*, 1983). There is evidence that the peripheral endings of sensory neurones release neurotransmitter during antidromic stimulation, to mediate some physiological processes (Bayliss, 1901; Lewis, 1927). Substance P fulfills several criteria for such a role (Lembeck & Gamse, 1985; Gamse *et al.*, 1981; Helme, White & Andrews, 1985). Since CGRP is a potential candidate as a neurotransmitter by virtue of its localization and pharmacological actions, we have investigated the effects of CGRP in skin, where sensory neurones are implicated in neurogenic inflammation. We have previously shown that the production of flare in human skin by peptides, such as substance P, is related to the ability of such peptides to release histamine *in vitro* (Foreman *et al.*, 1983). We therefore examined the histamine releasing action of CGRP.

## METHODS

### *Reagents*

Human calcitonin gene-related peptide, substance P and [D-Pro$^4$, D-Trp$^{7,9,10}$]SP$_{4-11}$ were obtained from Peninsula Laboratories Europe, St. Helens, U.K. The peptides were dissolved in acetic acid 0·1% v/v at a concentration of about 1 mM, and stored frozen under nitrogen at −20°C. Histamine, o-phthalaldehyde, benzalkonium chloride and HEPES were obtained from Sigma Chemical Co., Poole, U.K. All other reagents were of 'AnalaR' quality from B.D.H., Poole, U.K. Chlorpheniramine was purchased as 'Piriton' (Allen and Hanbury's, Ware, U.K.), and prilocaine was obtained ready for injection as 'Citanest plain' (Astra Labs, King's Langley, U.K.). Composition of the modified Tyrode solution was: NaCl 137 mM; KCl 2·7 mM; $NaH_2PO_4$ 0·4 mM; HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid) 20 mM; glucose 5·6 mM.

### *Responses in human skin*

The procotol for these experiments was approved by the appropriate Ethics Committee. The experiments were performed in healthy, non-atopic adult volunteers in the age range 30–40 years, who were taking no medication either at the time of the experiment or during the previous 2 weeks. The method employed has previously been described in detail (Foreman *et al.*, 1983). Briefly, peptides were diluted from a freshly thawed and neutralized stock solution into sterile sodium chloride solution, 154 mM, and were adjusted, if necessary, to a final pH of 7·0–7·2. Injections were made intradermally into the skin of the volar surface of the forearm, in a volume of 25 μl. No more than four injections were placed in each forearm to avoid interaction between adjacent responses, and the position of a particular injection at a given site was determined randomly.

At various times after the injection, the extent of the skin responses was measured in two directions at right angles and the area of the response was calculated from a mean diameter. Where appropriate, the intensity of the response was scored on a scale of 0 to 4.

### *Skin biopsy*

Sterile saline or CGRP (50 pmol) were injected intradermally into the skin on the volar surface of the forearm of a volunteer. One hour after injection, the site was infiltrated with lignocaine 2% w/v and a 3 mm punch biopsy was taken. The biopsy was fixed in formol saline, embedded in paraffin, and thin sections cut. The sections were stained with haematoxylin and eosin, and examined microscopically.

### *Measurement of histamine release*

Male Sprague-Dawley rats weighing 250–350 g were killed by asphyxiation in nitrous oxide and cervical dislocation. Peritoneal cells were obtained by washing the peritoneal cavity with 2 × 15 ml of sodium chloride solution (154 mM) buffered with HEPES (20 mM), and containing heparin (25 U/ml); the pH was 7·4. The cells were then centrifuged in 1 ml aliquots at 200 *g* for 5 min and then re-suspended in a modified Tyrode solution, to give a final volume of 0·5 ml after addition of drugs. Following 10 min pre-incubation at 37°C, peptides were added to the cell suspension to give an appropriate concentration, and after a further 10 min incubation at 37°C, 4·5 ml of ice-cold Tyrode solution was added to terminate the reaction. The cell suspension was centrifuged at 800 *g* for 5 min. The supernatants were removed for the assay of released histamine and the pellets were resuspended in Tyrode solution and heated to 100°C for 5 min to release residual histamine. Histamine released from the cells, and that released by heating the pellet, was assayed fluorimetrically by the method of Shore, Burkhalter and Cohn (1959), without the extraction step. Histamine release was expressed as a percentage of total cell histamine. Histamine release evoked by peptide was corrected for spontaneous release, which was determined on aliquots of cells not treated with any histamine-releasing stimulus.

## RESULTS

Human CGRP released histamine from rat peritoneal mast cells in a dose-dependent manner over the concentration range 2·5–10 $\mu$M (Fig. 1). Compared with substance P, CGRP was about fourfold less potent as a histamine-releasing agent. Limited availability of the peptide prevented further experiments, or extension of the range of CGRP concentration. Figure 1 also shows that both benzalkonium chloride (10 $\mu$M) and [D-Pro$^4$, D-Trp$^{7,9,10}$] SP$_{4-11}$ (10 $\mu$M) inhibited the mast cell responses to CGRP and substance P. At these concentrations, benzalkonium chloride and [D-Pro$^4$,-D-Trp$^{7,9,10}$]SP$_{4-11}$ did not themselves significantly increase spontaneous histamine release.

When injected intradermally into human skin, CGRP produced approximately circular wheal and flare responses similar to those produced by intradermal injection of substance P. The wheal and flare responses to CGRP were dose-related in the range 12·5–50 pmol (Fig. 2). CGRP was found to be more than fourfold less active than substance P in inducing flare, and much less active than substance P in producing a wheal. The flare response to CGRP, an area of erythema spreading for several centimetres from the point of injection, was maximal in size and intensity 3 min after injection. The flare response was not consistently seen in all subjects tested (no response to 50 pmol CGRP was observed in three out of 16 injection sites), and was fainter and

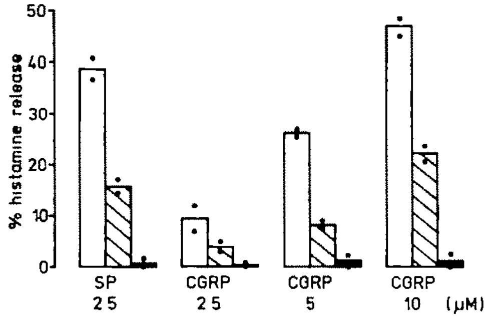

FIGURE 1. Inhibition by [D-Pro$^4$,D-Trp$^{7,9,10}$]SP$_{4-11}$ (SP-A) (10 $\mu$M) and by benzalkonium chloride (BAC) (10 $\mu$M) (final concentrations) of the release of histamine from isolated rat peritoneal mast cells induced by substance P (SP) and by CGRP. Mast cell suspensions were pre-incubated with the inhibitor 10 min prior to addition of releasing agent. The points are the results of two identical, separate experiments. ☐ Control, ▨ SP-A, ■ BAC.

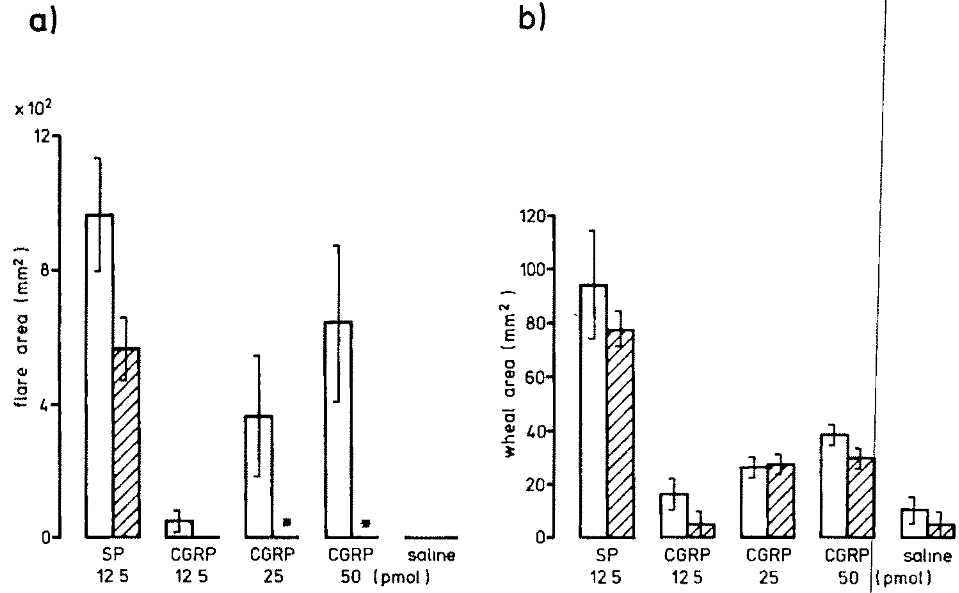

FIGURE 2. Effect of oral chlorpheniramine (16 mg given 1 h before the experiment) on substance P- and CGRP-induced flare (a) and wheal (b) responses in human skin. Flares were determined 3 min and wheals 12 min after intradermal administration of peptide. The vertical bars represent ±1 SEM ($n=4$) $\star = P<0{\cdot}05$, $t$-test. □ Control, ■ chlorpheniramine.

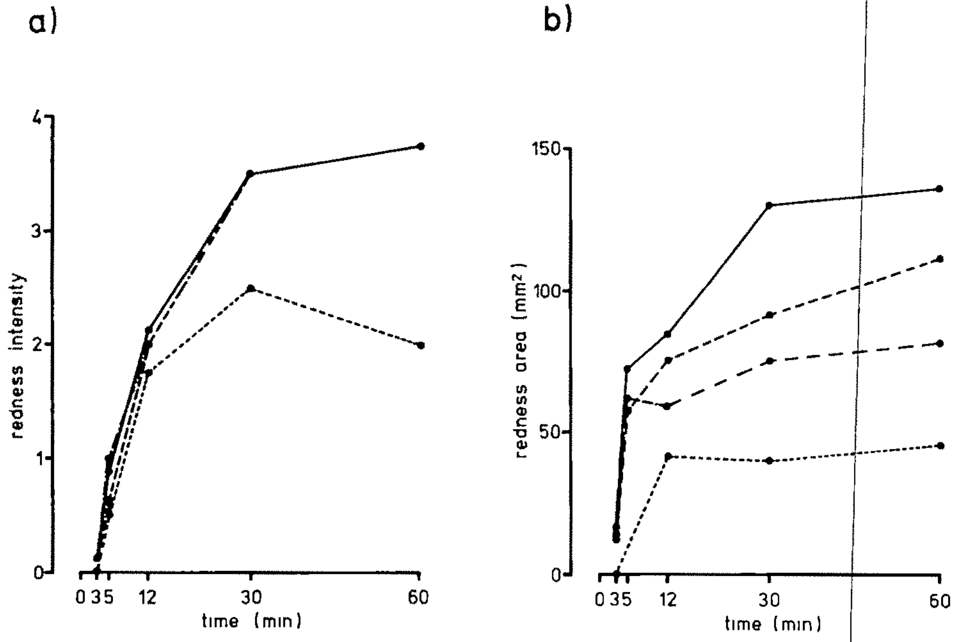

FIGURE 3. Time-course of the delayed erythema induced by CGRP in human skin. Redness intensity (a) was assessed on an arbitrary scale (0–4) whilst redness area (b) was calculated as described in the text. Each point is the mean of four separate experiments and error bars have been omitted for clarity. ---- 2·5 pmol CGRP, —— 12·5 pmol CGRP, – – – – 25 pmol CGRP, —— 50 pmol CGRP.

tended to fade more rapidly than that induced by substance P. These zero responses were included in the data for Figure 2. In contrast, the wheal response, an area of oedema localized to the site of injection, took longer to develop and was maximal in size 12 min after injection.

The flare responses to CGRP, but not the wheal responses, were markedly inhibited by pre-treatment of the subject with the $H_1$ histamine antagonist chlorpheniramine, 16 mg, taken orally 1 h before intradermal challenge (Fig. 2).

The flare responses to substance P and CGRP subsided rapidly after injection of the peptides, with little or no response remaining 30 min after injection. However, low doses of CGRP, which produced small and short-lived wheal and flare responses, gave rise to a slowly developing and intense erythematous response, which was surrounded by an area of pallor, around the site of injection (Figs 3 and 4). The delayed response to CGRP was seen at all doses of the peptide (2·5–50 pmol); it reached a peak about 1 h after injection and persisted for more than 3 h. The area of pallor was 2–5 mm wide; it was present in all experiments where the delayed erythema occurred, and developed with a time course similar to that of the erythema. The area of erythema was much smaller and much more intensely red than the flare response, and had a well defined boundary. The erythematous area produced by the highest dose of CGRP (50 pmol) also exhibited pseudopodia, or tracts of reddening, spreading away from the main response (Fig. 4). These too were bounded by an area of pallor.

Figure 8 (a) shows a micrograph of a section of skin from a site injected with saline. There is no evidence of the infiltration of granulocytes, monocytes, or other cells. Figure 8 (b) shows a high

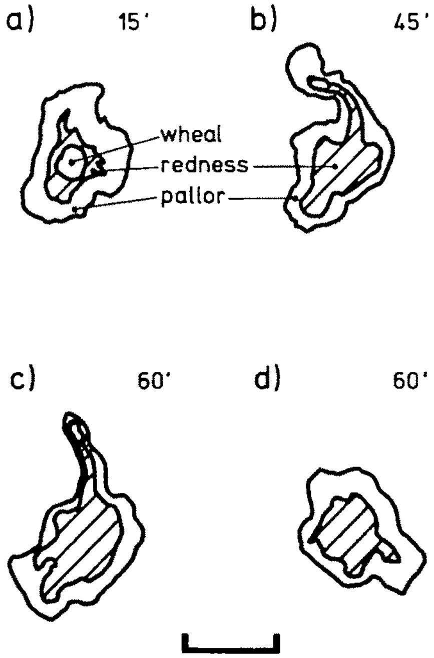

FIGURE 4. Tracings of the delayed responses of human skin to 50 pmol CGRP injected intradermally in a volume of 25 µl. a) and b) are tracings of the same injection site, 15 and 45 min respectively after peptide injection. c) and d) are tracings of two separate injection sites in the same subject, 60 min following CGRP injection, which were pre-injected with 25 µl saline c) or 25 µl 4% prilocaine d), 2 min before CGRP injection. The scale bar represents 2 cm.

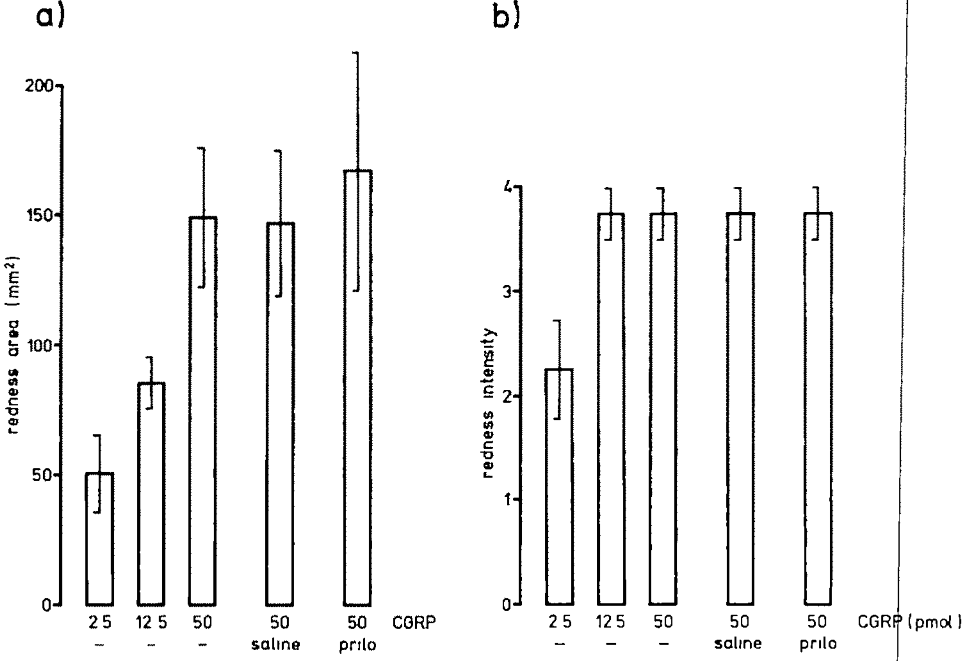

FIGURE 5. Effects of prilocaine (4% intradermally 2 min before CGRP injection) on CGRP-induced redness area (a) and intensity (b) in human skin, measured 60 min after peptide injection. The vertical bars represent ± 1 SEM ($n = 4$).

power view of a section of skin from a site injected with CGRP 50 pmol. It is evident, although the response is not large, that there is an increased infiltration of polymorphonuclear leukocytes in this section compared to the saline control. Substance P did not produce the delayed response. The erythema and surrounding pallor induced by CGRP were unaffected by pre-injection of local anaesthetic (Figs 4 and 5). A small, but statistically significant, reduction in the area of erythema was found in chlorpheniramine treated subjects 60 min after CGRP injection ($P < 0{\cdot}05$; Mann–Whitney test). This reduction was not detected 30 min post-CGRP (Figs 6 and 7). In the same experiments, chlorpheniramine reduced, by 42%, the area of flare produced in response to 12·5 pmol substance P.

## DISCUSSION

We have demonstrated that low doses of the recently discovered neuropeptide, CGRP, given intradermally, produced a marked erythema surrounded by an area of pallor. The vasodilator effects of CGRP have recently been observed (Brain *et al.*, 1985); however, no observations of a pallor induced by CGRP were mentioned in that report. Neither the vasodilation nor the pallor induced by CGRP were inhibited by chlorpheniramine pre-treatment or by local anaesthetic, suggesting that the involvement of histamine or of neuronal elements in these effects is unlikely. The small but significant reduction by chlorpheniramine, in the erythema after 60 min, suggests that the antihistamine may modify the local skin blood flow and alter the distribution of CGRP or its mediators. The slow onset of the vasodilation suggests that synthesis of a mediator or mediators is being induced. Arachidonic acid metabolites could be involved in the delayed-

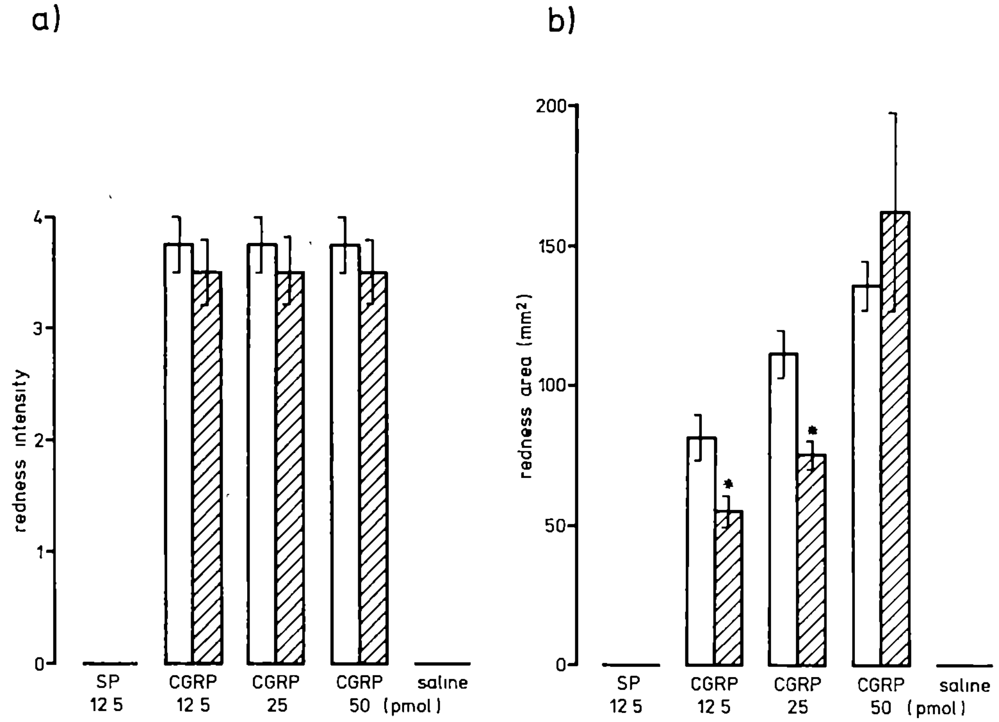

FIGURE 6. Effects of oral chlorpheniramine (16 mg given 1 h before injection) on CGRP-induced redness intensity (a) and area (b) in human skin, measured 60 min after peptide injection. The vertical bars represent ± 1 SEM ($n=4$) $^*P<0.05$, Mann-Whitney test. □ Control, ■ chlorpheniramine.

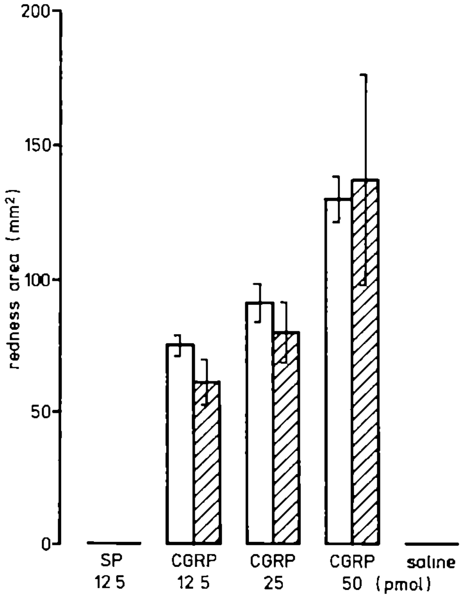

FIGURE 7. Effect of oral chloropheniramine (16 mg given 1 hr before injection) on CGRP-induced redness area in human skin, measured 30 min after peptide injection. The vertical bars represent ± 1 SEM ($n=4$). □ Control, ■ chlorpheniramine.

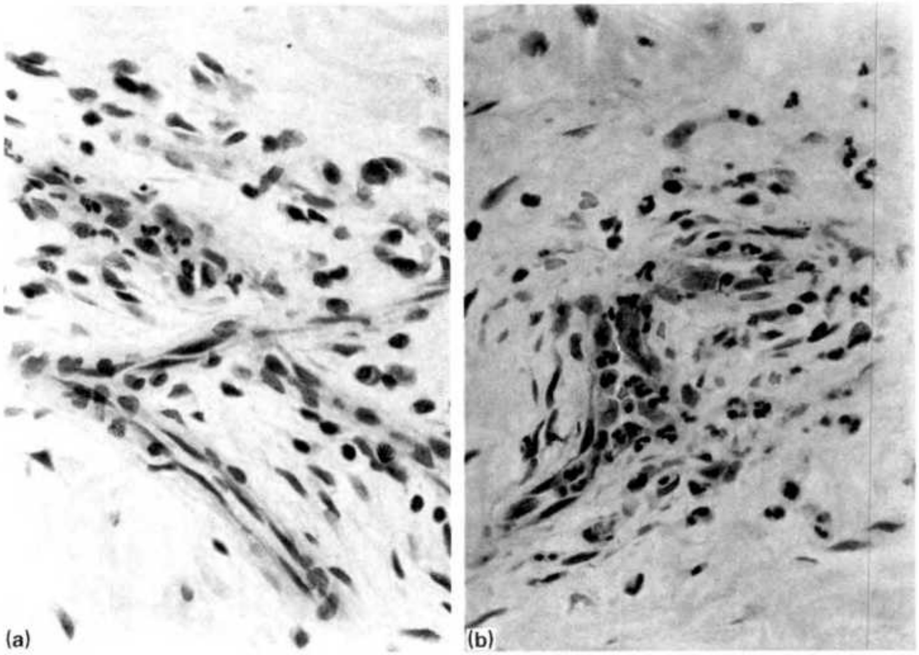

FIGURE 8. Photomicrographs of sections of human skin (Original × 400), obtained by punch biopsy and stained with haemotoxylin and eosin. (a) skin injected 1 h previously with saline, (b) skin taken from within the area of the erythematous response to 50 pmol CGRP, injected intradermally 1 h previously.

onset erythema, though probably not via an indomethacin-sensitive mechanism (Brain *et al.*, 1985). It is noteworthy that in rat aorta, relaxation induced by CGRP is endothelium-dependent (Brain *et al.*, 1985). The irregular spreading, by 'pseudopodia', of the delayed erythema response to CGRP is of interest and we concur with the view (Brain *et al.*, 1985) that this may represent migration of the peptide along lymphatics. That pallor around the erythema was also observed around these 'pseudopodia' suggests that this pallor is an integral part of the skin response to CGRP.

The results indicate that CGRP had increased cellular infiltration into the tissue 1 h after injection, when the erythematous response was fully developed. Therefore, it is conceivable that this peptide is either chemotactic itself or releases chemotactic material from other cells. The polymorphonuclear cells accumulating at the site may be involved in the generation of the delayed erythema response to CGRP by releasing vasodilator substances.

The slow onset and long duration of the erythematous response of human skin to CGRP makes this peptide an unlikely candidate as a mediator of the initial flare response induced by injury, or by the intradermal injection of histamine or substance P. However, it clearly suggests that this neuropeptide, which has been localized to sensory neurones (Rosenfeld *et al.*, 1983), may indeed be involved in longer-term vascular responses of skin to antidromic activation of these neurones, by injury for example. The wheal and flare responses to histamine and substance P have approximately similar time-courses (Foreman *et al.*, 1983).

The presence of the pallor around the erythema produced by CGRP may represent vasoconstriction or a reduced blood flow due to vessel compression by oedema. If CGRP is indeed released by injury, such reduced flow may act to limit the spread of an injurious influence at the site whilst vasodilation would facilitate the influx of neutrophils or the removal of a noxious stimulus by the blood stream.

A potent interaction of CGRP and substance P has been observed following their intrathecal injection in rats (Fischer *et al.*, 1985). Since CGRP has been shown to coexist with substance P, and is therefore likely to be co-released (Terenghi *et al.*, 1985; Mason *et al.*, 1984), it would be of interest to investigate this possible interaction.

We have demonstrated that concentrations of CGRP that are higher than those needed to induced the delayed and prolonged erythematous response, produced wheal and flare responses when injected into human skin. The flare is probably due to release of histamine from skin mast cells, since chlorpheniramine markedly attenuated this response in human subjects. CGRP also stimulated release of histamine from isolated rat peritoneal mast cells, which have been shown to be a useful model for predicting the flare-inducing potency of neuropeptides in human skin (Fewtrell *et al.*, 1982; Foreman *et al.*, 1983; Piotrowski, Jordan & Foreman 1983). Smooth muscle contractions induced by CGRP are attenuated by mepyramine (Tippins *et al.*, 1984) again suggesting that histamine is the mediator of these responses. That CGRP did not consistently induce flare in all subjects tested suggests that the skin mast cells may differ between individuals in their sensitivity to CGRP.

Benzalkonium chloride has previously been shown to inhibit histamine release induced by substance P (Read & Kiefer, 1979) and [D-Pro$^4$, D-Trp$^{7,9,10}$]SP$_{4-11}$ is a competitive antagonist of substance P for histamine release from rat mast cells (Piotrowski, *et al*, 1983). The inhibition by [D-Pro$^4$,D-Trp$^{7,9,10}$]SP$_{4-11}$ or benzalkonium chloride, of rat peritoneal mast cell histamine release induced by CGRP or substance P, suggests that these neuropeptides are acting at a similar site or sites to exert their secretory effects (Piotrowski *et al.*, 1983; Read & Kiefer, 1979).

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# Photosensitive dermatitis with actinic reticuloid syndrome: an immunohistological study of the cutaneous infiltrate

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## SUMMARY

Cryostat sections of skin biopsies from five patients with chronic photosensitivity dermatitis with actinic reticuloid syndrome (PDAR) have been examined immunohistologically by the alkaline phosphatase:anti-alkaline phosphatase staining technique using a panel of 24 monoclonal antibodies against lymphoid cells and their subsets. The lymphoid infiltrates in all cases had an essentially identical cellular composition, containing a mixture of T-lymphocytes, T-cell accessory cells (Langerhans cells) and other types of HLA-DR positive dermal macrophages. In two patients there was an excess of T-helper/inducer cells relative to T-suppressor cells, while in the other three patients the numbers of T-cells in these two subsets were approximately equal. Many of the infiltrating T-cells expressed activation (HLA-DR, interleukin-2 receptor) or proliferation (the Ki67 nuclear antigen, transferrin receptor) associated markers. These data indicate that a T-cell immune response is operative in cutaneous PDAR lesions.

Photosensitive dermatitis with actinic reticuloid syndrome (PDAR) is a chronic skin disease characterized by a severe, disabling sensitivity to ultraviolet radiation, and sometimes also to visible light (Ive *et al.*, 1969). The disease affects predominantly elderly men and usually has a benign clinical course, in spite of a histological similarity to cutaneous lymphomas of the mycosis fungoides type (Ive *et al.*, 1969).

The cause of the abnormal photosensitivity is obscure; although it has been proposed that a persistent T-cell immune response may play a role in its pathogenesis or maintenance (Thomsen, 1977). However, there are no data available concerning the nature of the massive lymphoid infiltrate found in PDAR, so that this concept remains unproved.

In the present study we have examined cutaneous PDAR biopsies immunohistologically by

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TABLE 1. Clinical details of five patients with chronic photosensitivity dermatitis with actinic reticuloid syndrome

| Patient | Age (years) | Sex | UVB sensitivity* | UVA sensitivity | Photo-patch tests | Positive patch tests | Duration of disease (years) | Therapy** | Present status§ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 75 | M | + | + | − | Chromium | 9 | Aza | A + |
| 2 | 73 | F | + | − | − | Thiuram-mix | 5 | MTX | A + |
| 3 | 63 | M | + | + | − | none | 9 | Aza | Ao |
| 4 | 47 | M | + | + | ND | Balsam of Peru; Colophony | 15 | Aza | A + |
| 5 | 73 | M | + | + | − | Paraphenylene-diamine; Fragrance-mix | 10 | Aza;MTX;P | D |

* + = positive; − = negative; ND = not done.

** Aza = azathioprine; MTX = methotrexate; P = prednisone.

§ A + = alive with active disease; Ao = alive in remission; D = dead.

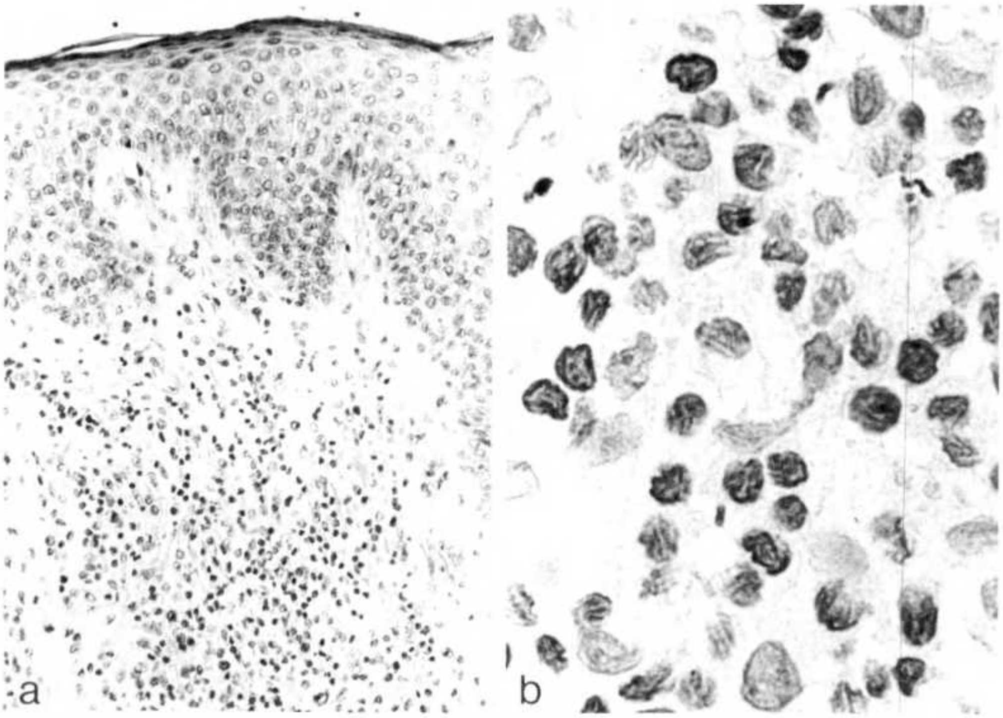

FIGURE 1. Dense dermal lymphoid infiltrate (a) in patient 1, showing single cell exocytosis (H & E, original × 250) and (b) containing many cells with a nuclear morphology resembling that seen in the Sézary or Lutzner cells (H & E, original × 1000).

the sensitive alkaline phosphatase: anti-alkaline phosphatase staining technique of Cordell *et al.* (1984) using a panel of 24 monoclonal antibodies against lymphoid cells and their subsets.

## METHODS

### *Patients*

Five patients with a clinical and histological diagnosis of PDAR have been studied. Their clinical data are summarized in Table 1. A representative example of the histopathological changes seen in a formalin fixed, haematoxylin and eosin stained skin biopsy is shown in Figure 1.

### *Processing of fresh tissue specimens*

Biopsies were frozen and stored in liquid nitrogen. 6 μm cryostat sections were air-dried overnight at room temperature, fixed in acetone for 10 min and either stained immediately or wrapped in aluminium foil and stored at −20°C until staining (Stein *et al.*, 1982). In the latter instance, sections were allowed to warm to room temperature prior to unwrapping.

TABLE 2. Monoclonal antibodies

| Antibody | Specificity | Reference or source* |
|---|---|---|
| Anti-Leu 1/Lyt 2 | Pan T-cell | BD/NEN |
| Anti-Leu 5/Lyt 3 | Pan T-cell (E-rosette receptor) | BD/NEN |
| Anti-Leu 4 | Pan T-cell | BD |
| Anti-Leu 3 | T-helper/inducer cells, macrophages | BD |
| Anti-Leu 2 | T-suppressor/cytotoxic cells | BD |
| Anti-Leu 7 | T-cells containing the fraction which acts as Natural killer cells | BD |
| OKT6 | Cortical thymocytes, Langerhans cells | Ortho |
| OKT9 | Transferrin receptor | Ortho |
| OKT10 | Haematopoietic precursor cells, activated peripheral cells | Terhorst *et al.* (1981) |
| Anti-Tac | Interleukin-2 receptor | Leonard *et al.* (1982) |
| Ki67 | Cell proliferation associated nuclear antigen | Gerdes *et al.* (1984) |
| B4 | Pan B-cell | CC |
| To15 | Pan B-cell | DAKO |
| R4/23 | Follicular dendritic cells | DAKO |
| To5 | C3b receptor | DAKO |
| EB11 | Macrophages | Bliss *et al.* (1985) |
| FMC32 | Macrophages | Hancock *et al.* (1983) |
| KB90 | Macrophages (p 150, 95) | D Y.Mason |
| CR3/43 | HLA-DR | D.Y.Mason |
| Anti-HLA-DR | HLA-DR | BD |
| Leu M1 | Granulocyte associated antigen (hapten X) | BD |
| Ki1 | Hodgkin and Reed-Sternberg cells | Stein *et al.* (1982) |

* BD = Becton Dickinson; NEN = New England Nuclear; Ortho = Ortho Diagnostics; CC = Coulter Clone; DAKO = Dakopatts.

TABLE 3. Reactivity of the infiltrating lymphoid cells in PDAR biopsies using monoclonal antibodies

| | Lymphocyte associated | | | | | Macrophage associated | | | Activation/proliferation associated | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient | Pan-T cells | T-helper cells | T-suppressor cells | Pan-B cells | NK cells | Langerhans cells | Tissue macrophages | FD cells | Anti-Tac | HLA-DR | OKT10 | OKT9 | Ki67 |
| 1 | + | + | – | – | – | +/– | +/– | – | +/– | + | – | +/– | +/– |
| 2 | + | + | – | – | – | +/– | +/– | – | +/– | + | – | +/– | +/– |
| 3 | + | +/– | +/– | – | – | +/– | +/– | – | +/– | + | – | +/– | – |
| 4 | + | +/– | +/– | – | – | +/– | +/– | – | +/– | + | – | +/– | – |
| 5 | + | +/– | +/– | – | – | +/– | +/– | – | + | + | – | +/– | +/– |

NK cell = natural killer cell, FD cell = follicular dendritic cell, + = labelling of a majority of the infiltrating cells, +/– = labelling of a proportion of the infiltrating cells, – = labelling of occasional or no cells.

### *Antibodies*

Details of the monoclonal antibodies used in this study are given in Table 2. Immune complexes of alkaline phosphatase and anti-alkaline phosphatase (APAAP complexes) were prepared as described previously (Cordell *et al.*, 1984). Unconjugated rabbit anti-mouse immunoglobulin was purchased from Dakopatts, Copenhagen, Denmark.

### *Immunostaining procedures*

Sections were stained by the alkaline phosphatase: anti-alkaline phosphatase (APAAP) method (Cordell *et al.*, 1984) using naphthol AS-MX (Sigma) and new fuchsin (Sigma) as substrate.

### *Control preparations*

Positive controls were performed by staining benign hyperplastic lymph nodes and tonsils; negative controls by omitting the primary monoclonal antibodies.

## RESULTS

The results from immunohistochemical investigation of cryostat sections of PDAR biopsies are summarized in Table 3. The composition of the cellular infiltrates was essentially identical in all cases. The lesions contained numerous T-lymphocytes reactive with three anti-pan-T-cell reagents (anti-Leu 5/Lyt 3, anti-Leu 1/Lyt 2 and anti-Leu 4) and negative with antibody OKT6

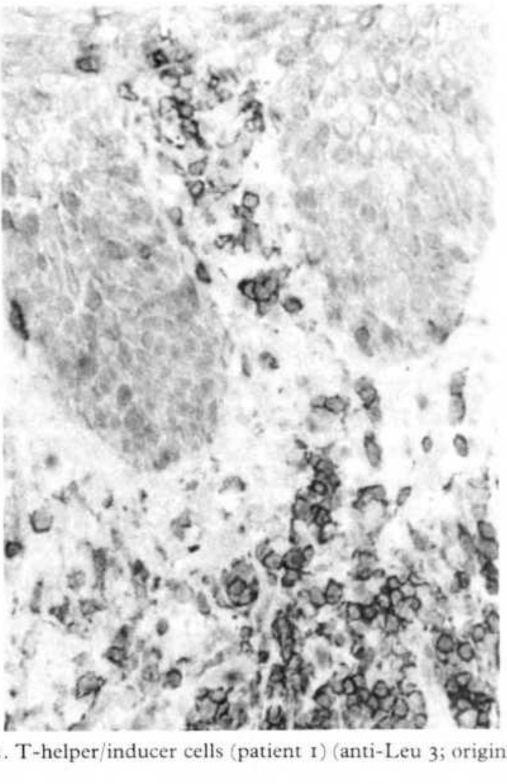

FIGURE 2. T-helper/inducer cells (patient 1) (anti-Leu 3; original × 250).

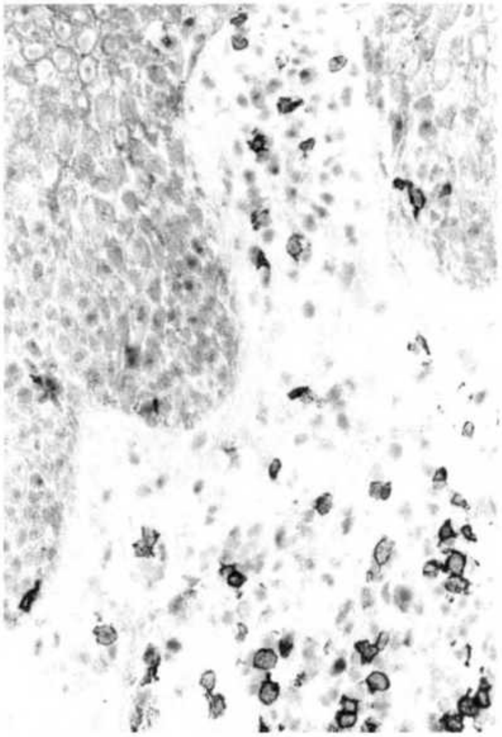

FIGURE 3. T-suppressor/cytotoxic cells (patient 1) (anti-Leu 2; original × 250).

directed against thymic cortical T-cells. In two patients (nos 1 and 2), the infiltrates showed a clear predominance of T-cells of the helper/inducer type (anti-Leu 3+). In these cases, T-suppressor/cytotoxic cells (anti-Leu 2+) were found only occasionally in the dermal or epidermal lymphoid infiltrate (Figs 2 and 3). The remaining three patients (nos. 3, 4 and 5), showed approximately equal numbers of T-helper and T-suppressor cells.

In all cases, a proportion of the infiltrating cells expressed markers associated with activation of T-cells, i.e. HLA-DR (Fig. 4) and interleukin-2 receptors (Fig. 5). A proportion of the cells also expressed markers associated with cell proliferation (transferrin receptor, the Ki67 nuclear antigen). OKT10-positive cells were not identified in any of the biopsies.

In addition to the T-lymphocytes described above, all the infiltrates contained non-lymphoid cells of dendritic morphology. These dendritic cells comprised Langerhans cells (OKT6+, HLA-DR+) and also other types of HLA-DR-positive, OKT6-negative dermal macrophages recognized by their labelling with the anti-macrophage reagents EB11, FMC32 and KB90 (Figs 6 and 7). Staining for HLA-DR also revealed that two patients (nos 1 and 5) showed focal anti-HLA-DR-positive deposits between the keratinocytes (Fig. 4).

B-lymphocytes (B4+, To15+), follicular dendritic cells (R4/23+, To5+), anti-Leu 7-positive cells (containing the cell fraction which acts as natural killer cells), or cells reactive with the Ki1 antibody (against a Reed-Sternberg cell associated antigen) were not identified in any of the biopsies. Staining with the anti-granulocyte antibody Leu M1 (anti-hapten X) was confined to scattered granulocytes present in the dermal infiltrate.

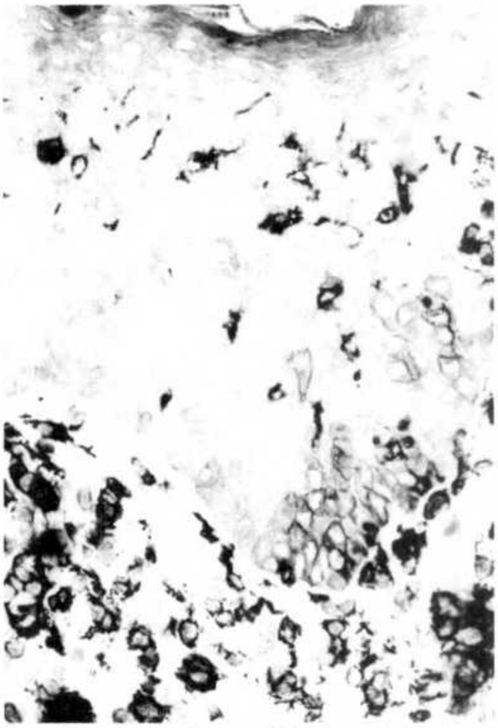

FIGURE 4. Anti-HLA-DR-positive cells (patient 1; original × 400). The number of HLA-DR positive cells clearly exceeds the number of Langerhans cells and macrophages (see Figs 6 and 7) indicating HLA-DR expression by the infiltrating T-lymphocytes shown in Fig. 2. Note also the focal HLA-DR-positive deposits between the keratinocytes.

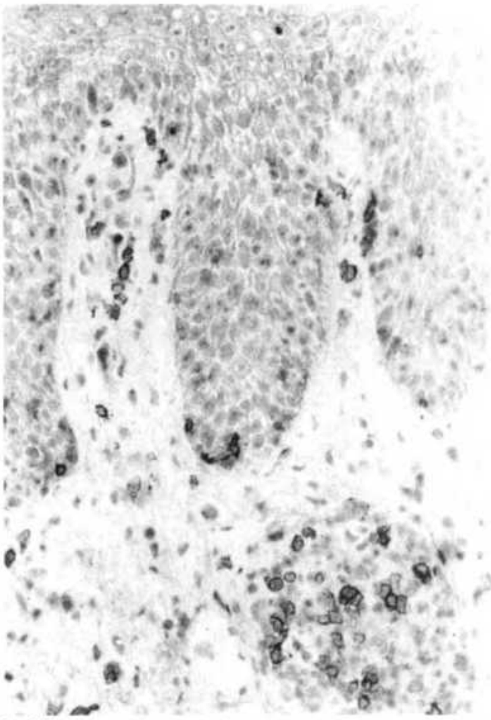

FIGURE 5. Interleukin-2 receptor-positive cells (patient 1; anti-Tac) (original × 250).

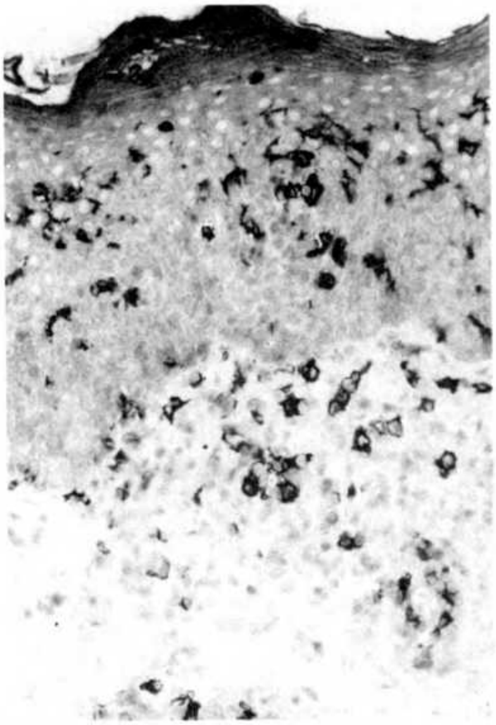

FIGURE 6. Langerhans cells in the epidermis and dermis (patient 1) demonstrated by staining with OKT6 (original × 250).

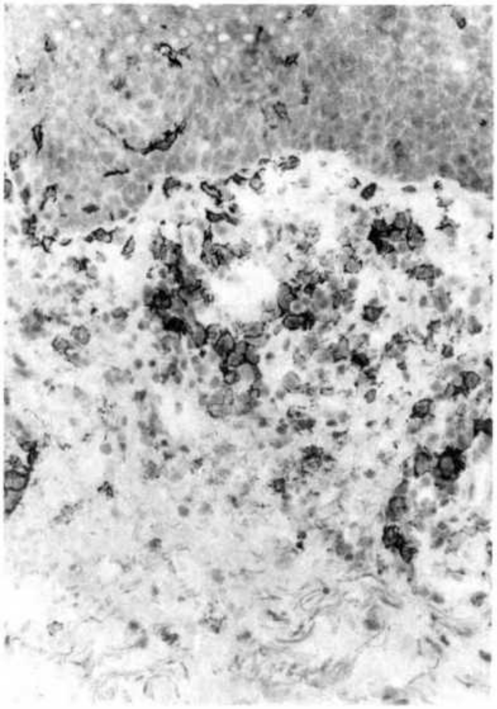

FIGURE 7. Macrophages in actinic reticuloid (patient 1) demonstrated by staining for the p150,95 molecule (KB90; original × 250).

## DISCUSSION

The results reported here demonstrate that the lymphoid infiltrates found in cutaneous PDAR biopsies have a mixed composition and contain T-lymphocytes in association with T-cell accessory cells (Langerhans cells) and other types of HLA-DR positive dermal macrophages. This supports the idea that an immune response of the T-cell type is operative in cutaneous PDAR lesions (Thomsen, 1977). The finding that the lymphoid cells in PDAR express markers linked to cell proliferation [the Ki67 antigen (Gerdes *et al.*, 1984), transferrin receptor (Terhorst *et al.*, 1981)] is in keeping with this view, since relatively rapid lymphoid cell turnover occurs predominantly in skin disorders associated with cutaneous immune responses (Ralfkiaer *et al.*, 1985b).

The fact that the cutaneous PDAR lesions often contain atypical lymphoid cells resembling the Lutzner or the Sézary cells (Ive *et al.*, 1969) does not constitute evidence against this suggestion, since Sézary-like cells have also been identified in patch test infiltrates (Meyer *et al.*, 1977). Furthermore, *in vitro* activated lymphoid cells may assume Sézary-like cytological features (Yeckley *et al.*, 1975), suggesting that the presence of these cells in cutaneous infiltrates is linked to the state of activation of the cells. This idea is in excellent agreement with the finding that many of the infiltrating T-cells in the present PDAR biopsies expressed activation-associated markers [HLA-DR (Reinherz *et al.*, 1979), interleukin-2 receptors (Leonard *et al.*, 1982)].

In recent studies of another photodermatosis (polymorphic light eruption), a predominance of activated (HLA-DR positive) T-cells similar to that found in this report has been identified (Moncada *et al.*, 1984; Muhlbauer *et al.*, 1983). On the basis of this finding it has been proposed that polymorphic light eruption results from an abnormal cutaneous immune response (Moncada *et al.*, 1984). However, HLA-DR positive T-cells are present in many skin disorders of widely different aetiology [e.g. atopic dermatitis, psoriasis, lichen planus, positive patch tests, chronic eczema and large plaque parapsoriasis (Ralfkiaer & Lange Wantzin, 1984; Ralfkiaer *et al.*, 1985a)], and accordingly are not necessarily indicative of an abnormal cutaneous immune response.

In PDAR, there is no evidence that abnormalities in the immune system have a primary pathogenetic role (Menter, McKerron & Amos, 1974). In fact, it has been suggested that the cause of the abnormal photosensitivity seen in this condition is an increased susceptibility of cutaneous fibroblasts to ultraviolet radiation (Giannelli *et al.*, 1983). This implies that the lymphoid infiltrate plays a role in the maintenance of the disease rather than in its initiation. Further *in vitro* functional analyses of the lymphoid infiltrate present in PDAR are necessary to fully elucidate this issue.

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# Arachidonic acid metabolism by polymorphonuclear leukocytes in psoriasis

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## SUMMARY

The metabolism of endogenous arachidonic acid by human polymorphonuclear leukocytes (PMNL) isolated from peripheral blood has been studied in 19 patients with chronic plaque psoriasis and 19 healthy controls. Using calcium ionophore A23187 as a stimulus, the PMNL synthesized leukotriene $B_4$ ($LTB_4$), 6-*trans*-leukotriene $B_4$, 12-epi-6-*trans*-$LTB_4$, and 5-hydroxy-6,8,11,14-eicosatetraenoic acid. There was no significant difference in the amounts of the products formed between the psoriatic and control groups. The elevated levels of $LTB_4$ that have been described in psoriatic skin may therefore be due to the PMNL infiltrate or to enhanced synthesis by another cell type. The reported increase in activity of the circulating PMNL in psoriasis does not appear to be due to increased 5-lipoxygenase activity in these cells.

Polymorphonuclear leukocytes (PMNL) are prominent in the histology of the psoriatic lesion, appearing early and possibly before the characteristic epidermal changes (Pinkus & Mehregan, 1966; Jablonska *et al.*, 1982). This has prompted studies to ascertain whether there is any intrinsic abnormality of the PMNL in psoriasis. Measurements of chemotaxis and chemokinesis (Wahba *et al.*, 1978; Kawohl *et al.*, 1980; Breathnach, Carrington & Black, 1981; Fraki *et al.*, 1983; Preissner, Schroder & Christophers, 1983), adherence (Sedgwick, Bergstresser & Hurd, 1980), phagocytosis (Wahba *et al.*, 1978), superoxide generation and degranulation (Sedgwick, Bergstresser & Hurd, 1981; Preissner *et al.*, 1983), and enzyme activities (Glinski *et al.*, 1984) have yielded conflicting results; where abnormalities have been found, they have often appeared to be secondary to active disease, rather than primary. The 5-lipoxygenase products of arachidonic acid (AA) metabolism, especially leukotriene $B_4$ ($LTB_4$), stimulate many of these PMNL activities, such as chemokinesis and aggregation (Ford-Hutchinson *et al.*, 1980), chemotaxis (Goetzl & Pickett, 1981), adhesion to endothelial cells (Cramer *et al.*, 1984), and degranulation and release of lysosomal enzymes (Hafstrom *et al.*, 1981; Feinmark *et al.*, 1981; Bokoch & Reed, 1981). It therefore seemed important to study arachidonic acid metabolism in the psoriatic PMNL.

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Recent work has demonstrated elevated levels of $LTB_4$ and other lipoxygenase products in psoriatic skin, using fluid collected in glass chambers from abraded plaque (Brain *et al.*, 1982a) and organic extracts of psoriatic scale (Camp *et al.*, 1983; Grabbe *et al.*, 1984). This could reflect increased production of these compounds by psoriatic keratinocytes, which do appear to have the necessary synthesising capacity (Hammarstrom *et al.*, 1979; Brain *et al.*, 1982b; Ziboh *et al.*, 1984), in which case other cell types, such as the PMNL, might be expected to show a similar metabolic imbalance.

A further reason for studying AA metabolism in the psoriatic PMNL is that some of the treatments used in psoriasis have been shown to act as lipoxygenase inhibitors *in vitro*. These include anthralin (Bedord, Young & Wagner, 1983), etretinate (Sircar & Schwender, 1983) and benoxaprofen (Walker & Dawson, 1979; Allen & Littlewood, 1982), although there is some doubt about the 5-lipoxygenase inhibitory activity of the latter (Masters & McMillan, 1984; Salmon *et al.*, 1984).

When human PMNL are incubated in the presence of the calcium ionophore A23187, they metabolize endogenous AA, predominantly via the 5-lipoxygenase pathway, to produce 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and subsequently $LTB_4$ (Borgeat &

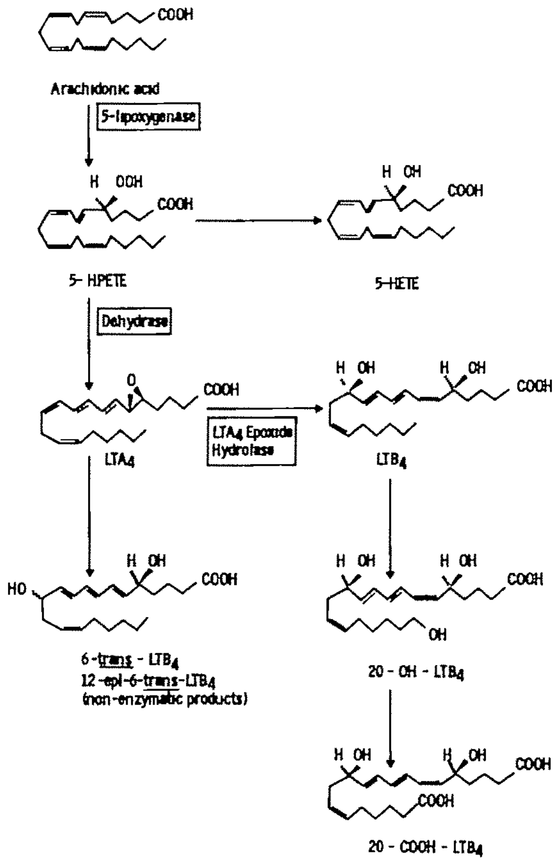

FIGURE 1. Metabolism of arachidonic acid by 5-lipoxygenase in human polymorphonuclear leukocytes.

Samuelsson, 1979; Sun & McGuire, 1984). Two non-enzymic isomers of $LTB_4$, 6-*trans*-$LTB_4$ and 12-epi-6-*trans*-$LTB_4$ are also formed during the course of this reaction (Fig. 1). In this study, the metabolism of endogenous AA has been compared in suspensions of purified PMNL from psoriatic and control subjects.

## METHODS

### *Patients*

Nineteen patients with chronic plaque psoriasis (9 male, 10 female, mean age 42 years) and 19 healthy controls (9 male, 10 female, mean age 33 years) were included in the study. Patients were selected who had no medical problems other than psoriasis and controls were healthy individuals who had no first-degree relatives with psoriasis. None of the patients or controls were on any medication (including the contraceptive pill) and all confirmed that they had not taken any preparation containing aspirin during the preceding 10 days. The patients were asked to discontinue all topical therapy except white soft paraffin for 3 days prior to venepuncture.

The extent of the psoriasis was noted in each case and ranged from under 5% to 70% of the surface area. None of the patients had erythrodermic or pustular psoriasis.

### *Preparation of PMNL suspensions*

Forty millilitres of venous blood were taken under standardized conditions into disodium EDTA (final concentration 4 mM). After centrifugation at 160 ***g*** for 10 min, the platelet-rich plasma was removed and centrifuged at 2300 ***g*** for 10 min, to yield platelet-free plasma. The lower layer containing red and white blood cells was then resuspended in about 3 ml of 0·9% (w/v) saline, and 6 ml layered onto 4 ml of Ficoll–Hypaque (density 1·077), followed by centrifugation at 400 ***g*** for 30 min. The supernatant layers, including a band containing mononuclear cells and platelets, were discarded, and the pellet of red blood cells and PMNL resuspended in a small volume of the platelet-free plasma. This was mixed with an equal volume of 6% (w/v) dextran (MW 110,000) in saline and the red blood cells allowed to sediment. After 40 min, the supernatant was removed and centrifuged at 400 ***g*** for 7 min, and the red cells remaining in the pellet of PMNL lysed by the addition of 0·2% (w/v) saline for 1 min, isotonicity being restored with 1·6% (w/v) saline. After further centrifugation, the PMNL were washed twice in Dulbecco's buffer (phosphate-buffered saline containing 0·5 mM $MgCl_2$ and 0·9 mM $CaCl_2$, pH 7·3) and finally resuspended in this buffer, with the volume adjusted to give a white cell count of $1{\cdot}5 \times 10^7$/ml in every case.

Films were prepared of the cell suspensions and stained by Leishman's method; the non-specific esterase method was also used in some cases. PMNL constituted between 88 and 100% of the total white cell count. Cell viability measured by the trypan blue exclusion test was greater than 95%.

### *Incubation of PMNL suspensions*

PMNL suspensions from all 38 patients and controls were incubated in 1 ml volumes with stirring at 37°C. After 5 min, 20 $\mu$l of calcium ionophore A23187 (Sigma Chemical Co), a 0·1 mg/ml solution in DMSO and water, was added, yielding a final concentration of 3·8 $\mu$M (final concentration of DMSO $<0{\cdot}2\%$). After a further 5 min, the reaction was terminated by acidifying the sample to pH 3·0 with 40 $\mu$l of 0·25M HCl and then adding 1·5 ml of ice-cold water. Prostaglandin $B_2$ ($PGB_2$, Sigma), 0·6 nmol made up in Dulbecco's buffer, was then added to each tube, to act as an internal standard.

### *Extraction of samples*

Each sample was extracted twice with freshly distilled ethyl acetate and the extracts from each subject pooled. The solvent was evaporated under nitrogen, and the residue redissolved in 600 $\mu$l of methanol:water (65:35), and stored at −50°C until analysis.

### *Separation and quantitation of products by HPLC*

Synthetic $LTB_4$, 6-*trans*-$LTB_4$, 12-epi-6-*trans*-$LTB_4$ and 5-HETE for use as standards were generously provided by Dr J.Rokach of Merck Frosst, Montreal, and $PGB_2$ was purchased from Sigma. The reverse phase HPLC system adopted was that described by Osborne, Peters & Meade (1983), as this has been shown to give good separation of the lipoxygenase metabolites of AA. A Waters pump (model M-6000A) and Techsphere 5 C18 Column, 12·5 × 0·5 cm (HPLC Technology Ltd) were used, with isocratic elution of methanol:water:acetic acid (65:35:0·06), pH 5·3, at a flow rate of 1 ml/min. Metabolites were detected by monitoring UV absorbance (Perkin-Elmer Spectrophotometer, model LC-55) at 270·5 nm to 20 min ($LTB_4$ and its isomers, and $PGB_2$) and then at 237 nm (5-HETE). A typical HPLC profile of ethyl acetate extracts from ionophore-stimulated PMNL is shown in Fig. 2.

In order to quantify the metabolites, the areas under the peaks were measured on a digitiser and the amount of product calculated by reference to the peak areas obtained with known amounts of standards. Recovery of $PGB_2$ was used to correct for losses during the procedure. The overall recovery rate for the 38 samples, as measured with $PGB_2$, was 56 ± 11% (mean ± S.D.). Values were expressed as nanograms of metabolite produced by 1·5 × $10^7$ cells in 5 min. Reproducibility was tested by measuring $LTB_4$ production in the same individual on five separate occasions over 4 weeks. Mean value was 214 ng/1·5 × $10^7$ cells/5 min, with a coefficient of variation of 17·0%.

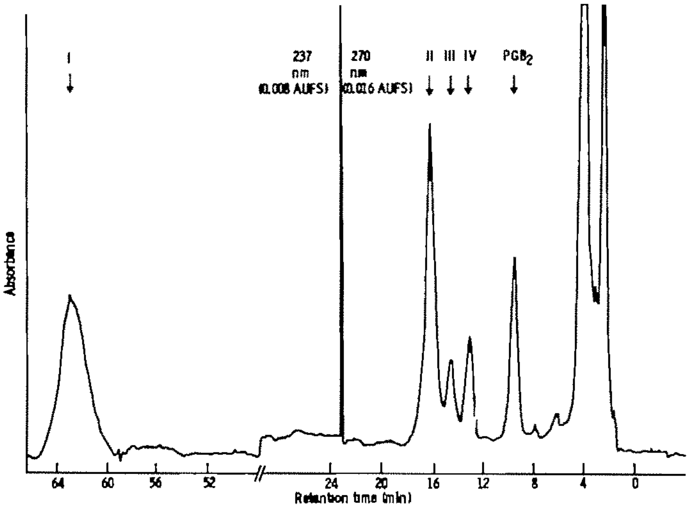

FIGURE 2 Representative HPLC analysis of the ethyl acetate extract from polymorphonuclear leukocytes incubated in the presence of A23187 for 5 min. Separation was by reverse-phase HPLC, with isocratic elution of methanol/water/acetic acid (65 35:0 06). The line shows UV absorption at 270 and 237 nm I = 5-hydroxy-6,8,11,14-eicosatetraenoic acid, II = leukotriene $B_4$; III = 12-epi-6-*trans*-$LTB_4$; IV = 6-*trans*-$LTB_4$; prostaglandin $B_2$ ($PGB_2$) was added as an internal standard.

### *Measurement of $LTB_4$ by radioimmunoassay*

In order to confirm the identity of $LTB_4$ and validate its quantitation by measurement of the UV absorbance peak area on HPLC, the fraction of solvent eluting off the column that contained $LTB_4$ was collected from 30 samples for analysis by radioimmunoassay (RIA). This was done using a commercially available $^3$H-$LTB_4$ assay reagents system (Amersham International). Aliquots (50 $\mu$l) of each solvent fraction were evaporated to dryness under nitrogen and then reconstituted in 5 ml of the RIA buffer. The amount of $LTB_4$ (ng) in the HPLC solvent fraction as measured by RIA was plotted against the amount (ng) calculated using the peak area (Fig. 3). This showed a good correlation between the values obtained using the two methods ($r = 0{\cdot}874$, $P < 0{\cdot}001$).

### *Statistical analysis*

The levels of 5-lipoxygenase products in psoriatics and controls were compared using the Mann-Whitney Test (MINITAB Statistical Software Package: Copyright Pennsylvania State University).

## RESULTS

The quantities of 5-lipoxygenase products synthesized by the ionophore-stimulated PMNL from control and psoriatic subjects are shown in Table 1. No statistically significant difference was seen for $LTB_4$, its non-enzymic isomers 6-*trans*-$LTB_4$ and 12-epi-6-*trans*-$LTB_4$, or 5-HETE, between the two groups. Nor was there any clear relationship between the level of $LTB_4$ and the extent of the psoriasis.

## DISCUSSION

As can be seen from the range of values in the table, there was a considerable variation between individuals in the capacity of their PMNL to metabolize endogenous arachidonic acid. This is in

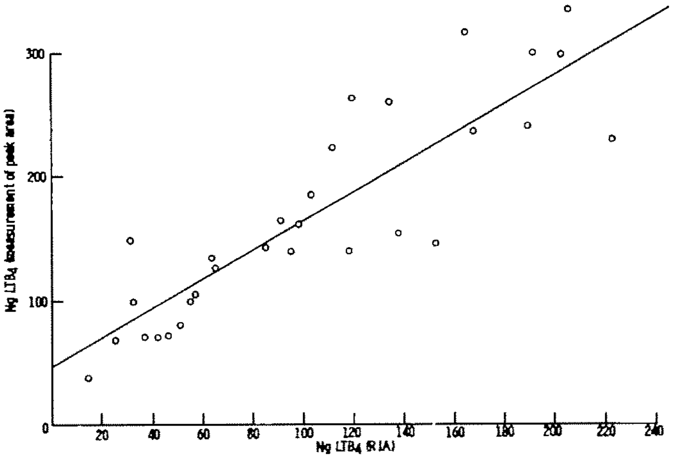

FIGURE 3. Relationship between the amount of leukotriene $B_4$ ($LTB_4$) in an extract of polymorphonuclear leukocytes as measured by the area under the UV absorbance peak on HPLC and by radioimmunoassay (RIA)

TABLE 1. Levels of 5-lipoxygenase products formed in PMNL stimulated with A23187, from normal and psoriatic subjects

| | Compounds formed (ng) per $1{\cdot}5 \times 10^7$ cells in 5 min* | | | |
|---|---|---|---|---|
| | $LTB_4$ | 6-*trans*-$LTB_4$ | 12-EPI-6-*trans*-$LTB_4$ | 5-HETE |
| Normal subjects ($n = 19$) | 207 (49–317) | 73 (23–154) | 89 (21–152) | 204 (63–480) |
| Psoriatic patients ($n = 19$) | 219 (113–442) | 91 (41–129) | 84 (44–189) | 183 (68–353) |

* The value given is the median, with the range quoted in brackets.

keeping with the findings of Borgeat & Samuelsson (1979). Despite this, no clear difference emerged in the arachidonic acid metabolism of psoriatic and control PMNL.

It has been argued that there might be a metabolically active subpopulation of PMNL in psoriasis, and these are the cells that infiltrate the skin, leaving the less active cells in the peripheral blood. Alternatively, the more active PMNL may be selectively lost during the purification procedure due to a slight difference in cell density (Phillips, Hosking & Shelton, 1983). However, the recovery of PMNL from whole blood during the preparation of suspensions was no less in the psoriatic group and any such effects are unlikely to totally obscure a real difference in metabolic capacity.

If a difference in the 5-lipoxygenase activity of psoriatic PMNL exists, a more likely reason for its being missed is that, in the present study, metabolites have only been measured at one point in time, i.e. 5 min after the addition of ionophore, when the levels are normally near their maximum (Shak & Goldstein, 1984; Sun & McGuire, 1984). It is known that $LTB_4$ is specifically and rapidly metabolized by the PMNL $\omega$-oxidation system to 20-OH- and 20-COOH-$LTB_4$ (Hansson *et al.*, 1981; Powell, 1984; Shak & Goldstein, 1984). If both the 5-lipoxygenase and $\omega$-oxidation enzymes were more active in the psoriatic PMNL, then the $LTB_4$ level at any one time might be unaffected. Alternatively, a highly efficient $\omega$-oxidation system may be able to deal with an increased rate of production of $LTB_4$, so that this is not apparent when levels are measured at one point in time.

The calcium ionophore A23187 is a potent stimulus of AA release and metabolism in the PMNL and might possibly obscure a modest difference in metabolic behaviour. However, we have been unable to elicit a substantial conversion of AA into lipoxygenase products in the PMNL using alternative stimuli, such as N-formyl-methionyl-leucyl-phenylalanine with cytochalasin B, zymosan-activated serum, or 0·25 mM AA, although these are all stimuli for degranulation in our system (unpublished observations). This is in accordance with the experience of others (Palmer & Salmon, 1983). It is possible that there is a difference in the metabolic capacity of the PMNL in psoriasis, but that it is seen only under the conditions for stimulation occuring *in vivo*.

With the above reservations, the results presented in this study demonstrate that there is no difference in AA metabolism in the circulating PMNL of psoriatic and control subjects. The findings of elevated $LTB_4$ in lesional psoriatic skin (Brain *et al.*, 1982a; Camp *et al.*, 1983; Grabbe *et al.*, 1984) could be due to the infiltrate of PMNL in the psoriatic plaque, even though these cells are normal with respect to their intrinsic capacity to produce $LTB_4$. It has recently been emphasized that migrating cells can change the eicosanoid profile of an inflamed tissue, as a result of their own metabolic activity (Moncada, 1985) Alternatively, the high $LTB_4$ levels may originate primarily from another cell type in the skin, such as the keratinocyte (Ziboh *et al.*, 1984).

Numerous studies have been performed on various aspects of PMNL function in psoriasis, with conflicting results. Wahba *et al.* (1978) demonstrated an increase in chemotaxis and phagocytosis, and Preissner *et al.* (1983) an increase in chemotaxis and enzyme release, by the psoriatic PMNL, and they suggested that this represented an intrinsic abnormality of the cell. However, other studies of PMNL chemotaxis, adherence, superoxide generation and enzyme release have either shown no abnormality at all in psoriasis (Breathnach *et al.*, 1981; Fraki *et al.*, 1983), or have found that increases in these functions are related to the extent and severity of the disease and are probably due to factors released from psoriatic plaque that circulate in the blood (Sedgwick *et al.*, 1980, 1981; Kawohl *et al.*, 1980; Glinski *et al.*, 1984).

On the basis of the evidence presented here, it is not possible to say that there is an intrinsic abnormality of AA metabolism in the psoriatic PMNL. The findings of elevated $LTB_4$ in psoriatic skin, and the functional abnormalities of the circulating PMNL, can be accounted for in other ways, without invoking a specific and primary metabolic abnormality in the PMNL.

## ACKNOWLEDGMENTS

We are very grateful to the Special Trustees of Nottingham University Hospitals who have financed this work; and to the Department of Medicine of the University Hospital for providing the laboratory space and facilities. We thank Dr A.W.Ford-Hutchinson for his help and advice and Dr J.Rokach for generously providing the HPLC standards (Merck Frosst, Montreal, Canada). We are also grateful to our colleagues in the Department of Dermatology for allowing us to include their patients in the study.

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# Increase in skin mast cells following chronic house dust mite exposure

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## SUMMARY

Application of inhalant allergens in high concentration to the mildly abraded skin of sensitive patients with atopic dermatitis gave rise to eczematous skin responses at 48 h. These lesions, infiltrated by basophils, eosinophils and mononuclear cells, are examples of cutaneous basophil hypersensitivity. Repeated application of allergen induced an increase in skin mast cells by 6 days, the mast cell hyperplasia replacing the earlier basophil infiltration. No electron microscopic evidence of mast cell heterogeneity among the recruited cells was found.

The naturally occurring lesions of chronic atopic dermatitis are infiltrated by mononuclear cells and show increased numbers of mast cells in the dermis (Mihm *et al.*, 1976). The lymphocytes present have both T helper and T suppressor phenotypes, suggesting a form of delayed hypersensitivity reaction (Leung *et al.*, 1983). The cell infiltrate usually does not include basophils or eosinophils, so that it is not typical of cutaneous basophil hypersensitivity, a form of delayed hypersensitivity reaction, which is characterized by these cells (Askenase 1977; Mitchell & Askenase, 1983). In renal allograft rejection (Colvin & Dvorak, 1974) and allergic contact dermatitis (Dvorak, Mihm & Dvorak, 1976) the early presence of basophils may be followed by an increase in mast cells and it remains unclear whether this is due to cell recruitment or to hyperplasia. It has been suggested that this same sequence could occur in the lesions of atopic dermatitis (Mihm *et al.*, 1976).

Patients with atopic dermatitis often have high levels of IgE antibody and positive immediate hypersensitivity to common inhalant allergens (Chapman *et al.*, 1985). We have previously demonstrated that these same inhalant allergens applied to the skin for 48 h can induce a delayed eczematous response with microscopic features of cutaneous basophil hypersensitivity (Mitchell *et al.*, 1982; Mitchell *et al.* 1984). In these lesions mast cell numbers were not increased. In

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the present study, we have demonstrated that repeated administration of allergen at high concentration to the same site results in the development of eczematous lesions in which a basophil infiltrate is replaced by a mast cell infiltrate. On electron microscopy the recruited mast cells were morphologically the same as the mast cells normally resident in the skin.

## METHODS

### *Patients*

Five patients who had suffered from atopic dermatitis since infancy were studied while in remission, using a modified patch test technique. All had suffered from severe and extensive skin inflammation and had positive immediate skin test reactions to *Dermatophagoides pteronyssinus* extract. Four also had bronchial asthma. The mean age was 25 years (range 20–30) and one patient was female. The experiments reported here were approved by the Northwick Park Hospital Ethical Committee.

### *Allergens*

Antigen $P_1$ was purified from an extract of *D. pteronyssinus* culture as previously described (Chapman & Platts-Mills, 1980). Ragweed allergen was obtained from Center Laboratories (North Carolina, U.S.A.). For patch tests, a 2 × 2 cm area of skin was gently abraded with a scalpel blade, removing the upper layers of the epidermis. The sites included the flexor aspect of the forearm, the outer aspect of the upper arm, and the back, medial to the scapula. Control patch test sites were always placed in a comparable position. Allergen diluted to 10 $\mu$g/ml (or less) in normal saline (0·5 ml) was applied to a pad of sterile gauze, occluded with plastic and retained in position using hypoallergenic tape. After 2 days the sites were inspected, visually graded and either biopsied or had the same quantity of allergen applied for a further 2 days. Allergen application was repeated at 2-day intervals up to 8 days.

### *Biopsies*

Biopsies were taken after 2, 6 and 10 days, under local anaesthetic using a 4-mm disposable skin biopsy punch. Biopsy specimens were fixed in Karnovsky's solution for 24 h, washed in four changes of 0·1M sodium cacodylate buffer, pH 7·4 at 4°C, and dehydrated in varying concentrations of alcohol. Subsequently, these specimens were embedded in glycol methacrylate and 1–2 $\mu$m sections were cut and stained with haematoxylin and eosin (H&E), a modified Giemsa technique and with Azure A. All sections were examined under a × 100 oil immersion lens, and infiltrating cells were identified and systematically counted over the whole section. For electron microscopy, specimens fixed in Karnovsky's solution for 24 h were post-fixed in osmium tetroxide, dehydrated through graded acetone and embedded in Spurr resin. Sections (1 $\mu$) were stained with toluidine blue, and thin sections were collected on copper grids, stained with uranyl acetate and Reynold's lead citrate and viewed in either a Philips 300, or JEOL 1200 electron microscope.

Morphometric measurements of the section widths and of the percentage of scrolled areas in the mast cell granules were performed using a Joyce-Loebl Magiscan I Image Analysis System. The widths of the light microscope sections were measured by drawing the maximum width of the H&E section onto its image on the Magiscan screen with a light pen and allowing the computer to measure the length of the line. To measure the percentage area of mast cell granules occupied by scrolls, very pale EM prints were prepared and the scrolled areas were hand shaded with black ink in order to give enough contrast for the Magiscan to differentiate the scrolled from the unscrolled areas.

## RESULTS

All patients studied were known to have a high IgE level (geometric mean 5617 IU/ml; range 2900–9900) and specific IgE antibody to antigen $P_1$ (geometric mean 203 BA units/ml; range 19–768) (Chapman & Platts-Mills, 1980) and had been found to give an eczematous patch test response to antigen $P_1$, the major house dust mite antigen. As had been shown previously, these reactions were seen at day 2 at sites where antigen $P_1$ (10 μg/ml) was applied but not at control sites where saline alone was applied. The positive lesions were characterized by the presence of erythema, papules, induration and exudation. In four patients the re-application of antigen $P_1$ every 2 days gave rise to a gradual change in the character of the lesions with less erythema, but

TABLE 1. Numbers of infiltrating cells in patch test sites[*]

| Patient[†] | Antigen[‡] | Day[§] | Basophils | Mast cells | Eosinophils | Mononuclear cells |
|---|---|---|---|---|---|---|
| 1 | Control | 2 | 7 | 70 | 15 | 239 |
| | $P_1$ | 2 | 66 | 70 | 972 | 1445 |
| | $P_1$ | 6 | 33 | 107 | 276 | 772 |
| | $P_1$ | 10 | 7 | 149 | 130 | 600 |
| 2 | Control | 2 | n.d. | n.d. | n.d | n.d. |
| | $P_1$ | 2 | 17 | 88 | 559 | 854 |
| | $P_1$ | 6 | 3 | 103 | 210 | 506 |
| | $P_1$ | 10 | 5 | 145 | 117 | 410 |
| 3 | Control | 2 | 6 | 111 | 1 | 634 |
| | $P_1$ | 2 | 38 | 129 | 96 | 988 |
| | $P_1$ | 6 | 36 | 139 | 219 | 1053 |
| | $P_1$ | 10 | 13 | 115 | 36 | 561 |
| 4 | Control | 2 | 1 | 49 | 7 | 206 |
| | $P_1$ | 2 | 26 | 49 | 310 | 630 |
| | $P_1$ | 6 | 34 | 138 | 837 | 529 |
| | $P_1$ | 10 | 18 | 144 | 65 | 341 |
| 5[¶] | Control | 2 | 0 | 24 | 1 | 141 |
| | $P_1$ | 1 | 33 | 49 | 59 | 606 |
| | $P_1$ | 2 | 52 | 39 | 376 | 482 |
| | $P_1$ | 3 | 40 | 48 | 60 | 1211 |
| | $P_1$ | 7 | 17 | 46 | 116 | 374 |
| 1[†] | Ragweed | 2 | 1 | 56 | 17 | 415 |
| | $P_1$ 0·1 μg | 2 | 5 | 62 | 56 | 476 |
| | $P_1$ 1 μg | 2 | 17 | 55 | 82 | 523 |
| | $P_1$ 10 μg | 2 | 26 | 65 | 155 | 781 |

[*] Histological sections examined were comparable in width. All infiltrating cells and resident mast cells in the dermis were counted.

[†] Patients 1–4 had repeated antigen $P_1$ application. In patient 1 a further study was done on day 2 on a separate occasion, when antigen $P_1$ was applied at different concentrations for 48 hours prior to biopsy.

[‡] Antigen $P_1$ was used at 10 μg/ml unless otherwise stated Ragweed allergen was used at 10 μg/ml in patient 1.

[§] Number of days between first application of antigen and subsequent biopsy

[¶] Patient 5 had allergen applied to different sites at day 0 and left *in situ* for varying time periods prior to biopsy.

n.d., Not determined.

further induration and crusting becoming apparent. By day 10 the centre of the lesions had become rather pale and was surrounded by erythema and scaling. In the case of one patient, antigen $P_1$ was applied on a single occasion and the lesions appeared maximum at day 3 and had almost disappeared by day 7 with little erythema, few papules and no oedema or crusting. All subjects reported increased systemic itching associated with repeated application of patches over 10 days, particularly noticable at days 2 and 4.

Biopsies of parallel allergen patches were taken at 2, 6 and 10 days. The cellular infiltrate present in all subjects at day 2 was similar to that previously reported, and included basophils, eosinophils, mononuclear cells and neutrophils (Table 1). The number of these cells was maximal between day 2 and day 6 (Table 1; Fig. 1). Mast cells, however, behaved differently. Over the 10-day period of repeated application, a greater than 50% expansion in mast cell numbers developed (Fig. 1). As is evident from Table 1, this pattern was seen in three out of four subjects, whereas in the fourth a minimal increase in numbers was associated with a particularly large number of skin mast cells in the original and the control biopsies. In contrast, a single application of allergen to each site in one patient produced an increase in basophils, eosinophils and mononuclear cells but did not induce any increase in mast cells over 7 days (Table 1).

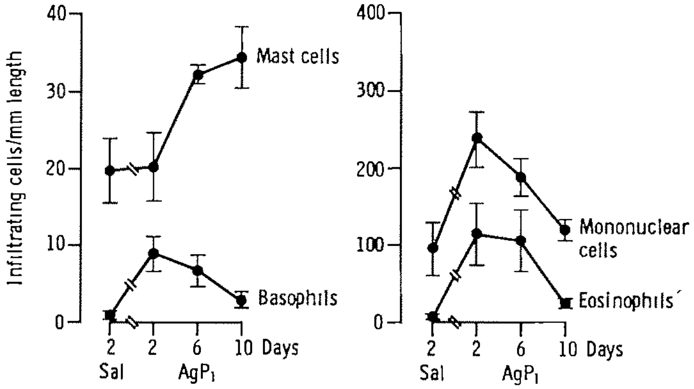

FIGURE 1. Number of infiltrating cells per unit (mm) length in patch test sites repeatedly exposed to antigen $P_1$ (days 2, 6 and 10) and saline (day 2). Results expressed as mean ± s.e.

TABLE 2. House dust mite antigen $P_1$ levels in patients' homes*

| | Antigen $P_1$ μg/g fine dust | | |
|---|---|---|---|
| Patient | Mattress | Bedclothes | Bedroom floor |
| 1 | 64 1 | 62 6 | 88 0 |
| 2 | 13 5 | 38·5 | 15 6 |
| 3 | 164 2 | 41·8 | 383 8 |
| 4 | n.d. | 22·8 | 28 6 |
| 5 | 94 3 | 200 0 | 561 6 |

*Antigen $P_1$ measured by vacuuming particular sites, collecting the dust samples, extracting and analysing using a double antibody inhibition radioimmunoassay.
n.d., Not determined.

Recruitment of other cell types in this experiment was comparable to that seen with repeated patch testing.

The concentration of antigen $P_1$ applied was large in comparison with that required to elicit immediate hypersensitivity. However, these patients were exposed to high levels of antigen $P_1$ in their homes (Table 2). A dose response on one patient showed a progressive increase in basophil and eosinophil infiltration from 0·1–10 μg/ml (Table 1). In addition, as was also reported previously, an antigen to which the patient had not been exposed, i.e. Ragweed, failed to induce significant cell infiltration (patient 1; Table 1).

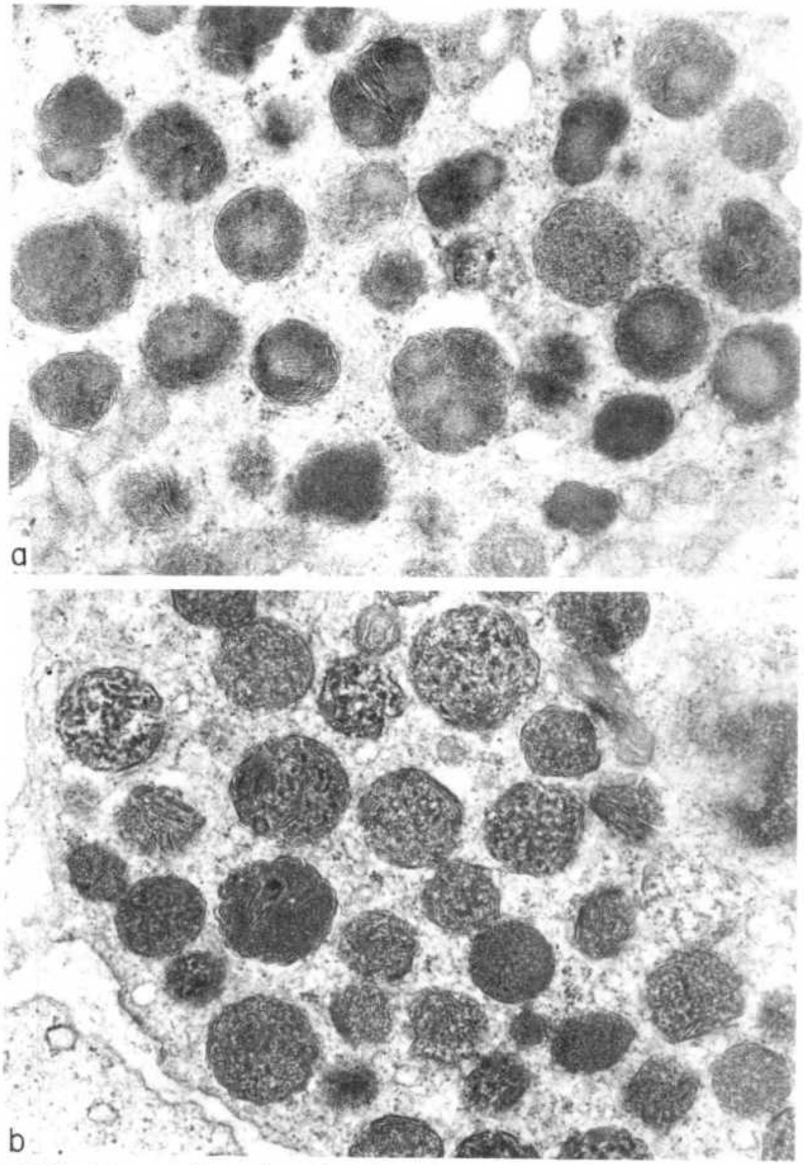

FIGURE 2. (a) Typical mast cell granules at day 2 from saline patch test site (Original × 34,020). (b) Mast cell granules from antigen $P_1$ site biopsied after 10 days repeated application. The granules show a variegated texture and prominent scroll structures (Original × 34,020).

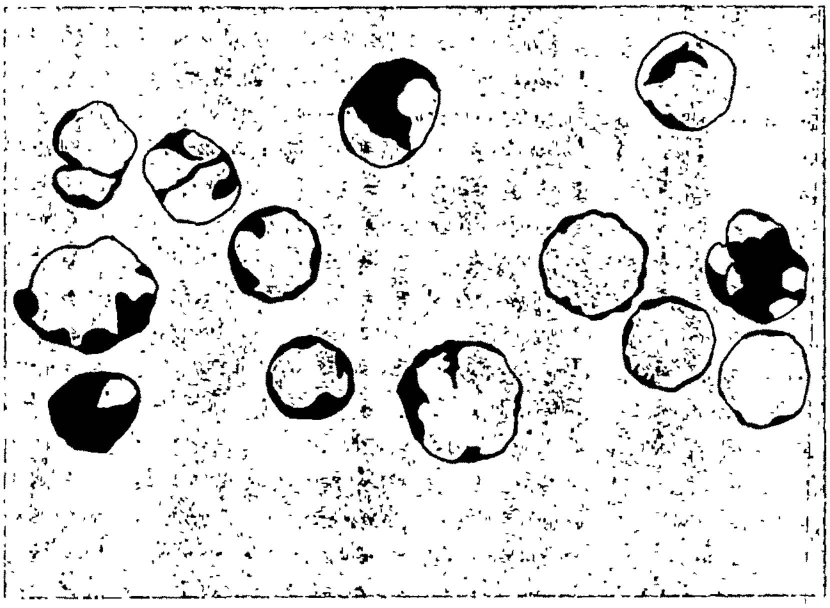

FIGURE 2. (c) Pale print of electron micrograph has been hand shaded with black ink to define areas containing scroll structures for measurement by the magiscan (Original × 34,020).

An electron microscopic analysis of mast cells in the lesions biopsied at different time points was performed. It was not possible to identify any definite morphological differences in the mast cells present in antigen $P_1$ or saline test sites at day 2, day 6 and day 10 (Fig 2a and b). However as some mast cell granules in the day 10 lesions did appear to have more prominent scroll structures, a large number of the granules (117) were analysed on the Magiscan Image Analysis System. There was no difference between the percentage area of granules occupied by scrolls in day 2 and day 10 lesions (42% versus 39%) (Fig. 2c).

## DISCUSSION

Detailed study of the morphology of inhalant allergen induced patch test reactions has shown that early basophil infiltration is subsequently replaced by an increase in mast cells. In previous human studies of contact dermatitis, similar results have been obtained, with basophil infiltration being replaced by inflammation in which mast cells become a feature (Dvorak *et al.*, 1976). We have found that to induce this change in patients with atopic dermatitis it was necessary to apply allergen repeatedly to the same skin site for 1 week or longer. Such repeated application closely mimics the natural exposure to house dust mite in the home where patients' skin is in direct contact with high concentrations of allergen for prolonged periods each day or night (Tovey, Chapman & Platts-Mills, 1981). The failure of a single application of allergen to induce mast cell changes further supports the need for chronic exposure. The concentration of allergen used in our tests was in keeping with both the home exposure levels, and the antigen concentrations required to induce cutaneous basophil hypersensitivity in experimental guinea-pigs (Askenase, 1977). We have demonstrated that low concentrations of allergen give rise to a lesser degree of cellular infiltration. It is of interest that the chronic lesions of atopic dermatitis are not infiltrated by basophils and eosinophils, but do have increased numbers of mast cells, Again in the patch test lesions, eosinophils were decreasing in numbers at a time when mast cells were increasing. Thus, repeated application of allergen to the skin results in lesions that macroscopically and in terms of the microscopic cellular infiltrate, resemble chronic lichenified ezcema.

It has now become clear that tissue mast cells are probably not a homogeneous population, since differences based upon fixation and staining, and functional characteristics have been identified in rodents and in man (Strobel, Miller & Ferguson, 1981; Metcalfe, 1983). We considered the possibility that the increase in mast cells produced in the skin would represent infiltration by a subpopulation of these cells. However, detailed ultrastructural examination failed to show any striking differences in mast cells present on day 2 and day 10. Our observations (E.B.Mitchell *et al.*, unpublished) on human skin and intestinal mast cell granules suggest that there is more active granular scrolling in mucosal than in connective tissue mast cells, although this may represent differences in the degranulation cycle rather than heterogeneity. We carefully analysed the mast cell granules in the patch test biopsies for the percentage area of the granule occupied by scroll-like structures, but found no significant differences. We conclude that the mast cell expansion that occurs does not involve cells that differ structurally. This, of course, does not rule out a functional heterogeneity.

Basophil recruitment in experimental animals is a regulated phenomenon, predominantly associated with T-cell function (Askenase, 1977; Mitchell & Askenase, 1982). In man it is apparent that basophils will exhibit chemotaxis to lymphocyte products and activated complement components (Lett-Brown, Boetecher & Leonard, 1976). We have demonstrated that patients with house dust mite allergic disease have sensitized T-cells and that allergen exposure *in vitro* can induce those sensitized lymphocytes to produce a basophil chemotactic activity, which appears to be due to a factor with a molecular weight of approximately 20000 daltons (Mitchell *et al.*, 1984). The expansion in mast cell numbers that develops with chronic antigen exposure may be due to cell differentiation or replication (Galli & Dvorak, 1984). In experimental animals a particular subset of mast cells has been shown to differentiate under T lymphocyte control (Davidson *et al.*, 1983). The basophils and mast cells that are recruited or that differentiate at a chronic allergic inflammatory site presumably have an effector function. This may be one of alteration in vascular tone or permeability and may be dependent upon the production of mediators via an IgE dependent pathway (Schleimer *et al.*, 1983). However, new evidence in mice suggests that the immune response controlling mast cells can also involve T-cells (Askenase *et al.*, 1983). An antigen specific T-cell factor that results in mast cell mediator release has been demonstrated. Furthermore, a number of inflammatory mediators such as interleukin-2 and gamma interferon are known to alter mast cell mediator release (Lett Brown *et al.*, 1981). It is likely therefore that the cellular inflammation that occurs following the application of allergen to the skin involves T-cells as well as IgE antibody and that T-cell regulation including the switch from basophil to mast cell infiltration is in progress. It is possible that mediators released from basophils and mast cells, e.g. histamine, have a regulatory role as well as being vasoactive and may influence the inflammatory response that develops in the skin (Rocklin *et al.*, 1978). Of interest is the observation that, in spite of continued allergen exposure, the increased numbers of mast cells present do not appear to be degranulated or to continue active recruitment of eosinophils as they had done earlier following allergen application. Therefore, the exact role of this cell type in chronic inflammation remains unclear.

From our observations we would suggest that the immunological process in atopic dermatitis can be explained by cellular immune mechanisms induced by allergen exposure. These mechanisms involve a form of delayed hypersensitivity response and presumably in the early stages, overlap with the mast-cell mediated late phase responses (Solley *et al.*, 1976). It is important to remember that there are many different changes in the skin of these patients, including biochemical and neurovascular changes. It is not at all clear whether these changes are primary or could be secondary to prolonged inflammation. If allergens can be implicated in the inflammation of atopic dermatitis, attempts at avoidance are necessary and in the case of the house dust mite it is now clear that marked reductions in allergen levels throughout the home can be achieved (Mitchell *et al.*, 1985).

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# A direct gravimetric technique for measuring sebum excretion rate

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## SUMMARY

The standard gravimetric method for measuring sebum excretion rates involves ether extraction of the collected sebum. We have evaluated a more direct method in which the collecting papers were pre- and post-weighed in glass beakers under conditions of stable temperature and humidity. Weights were adjusted allowing for control weight gain or loss, due mainly to day to day changes in weight room humidity. The direct method compared favourably with the ether extraction method in determining: (1) sebum collected from 43 subjects ($r = 0{\cdot}991$, $P < 0{\cdot}001$), (2) left versus right forehead comparisons in 19 subjects (direct method: $r = 0{\cdot}966$, $P < 0{\cdot}001$; ether extraction method: $r = 0{\cdot}963$, $P < 0{\cdot}001$), and (3) recovery of sebum from 32 premeasured standards ($r = 0{\cdot}997$, $P < 0{\cdot}001$). The findings demonstrate the accuracy of the direct gravimetric procedure which is also simpler, safer and less expensive than methods currently in use.

Since its introduction by Strauss & Pochi (1961), the gravimetric method for quantifying sebum excretion has been widely employed (Strauss & Pochi, 1961; Cunliffe & Shuster, 1969). In this technique, sebum is collected onto cigarette paper from a measured area of skin over a prescribed period of time. The sebum is extracted from the paper with ether which is evaporated off. The sebum residue is then weighed and the sebum secretion rate (SER) calculated.

A more direct method of simply pre- and post-weighing the collecting papers has not been reported, possibly because of concern that extraneous factors such as moisture and corneocyte shedding might adversely affect the results.

We now report such a method which, when followed carefully, accurately assesses weights of sebum excreted from skin.

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## METHODS

### *Equipment and materials*

(1) Cigarette papers cut into 18 × 3·5 cm strips and folded twice to produce 4·5 × 3·5 cm rectangles (four sheets in thickness).

(2) Small glass beakers, each etched with identifying numbers.

(3) Forceps.

(4) A Sartorius 2004 MPG electronic analytical balance which reads to 0·01 mg with a weighing range of 166 g and s.d. of 0·02 mg.

(5) A weighing room with relatively constant temperature (range 23–25°C) and humidity (range 34–42%).

### *Direct technique*

The technique employs the following steps.

(1) Clean beakers are placed in a clean tray, with a maximum of 10 beakers per tray (from this point until after the final weighing the beakers are handled only with forceps).

(2) Two papers are placed upright into each beaker.

(3) The entire tray is left in the weighing room for at least 2 h, but not more than 24 h, to equilibrate with room humidity.

(4) The beakers are then preweighed. (During the weighing, humidity must be stable and is monitored with a humidistat.) (a) The tray of beakers is placed immediately adjacent to the open balance before weighing. (b) Using ether cleaned forceps for handling, the beakers are weighed consecutively until each beaker in the batch is within 0·05 mg of its previous weight. (c) After the last weighing of the last beaker, the first beaker is reweighed to recheck that its weight (as a reflection of room humidity) has not changed beyond the ±0·05 mg allowance. If this limit is exceeded, the beakers are reweighed.

(5) When the papers are needed, the tray of beakers is taken to the patient collection area.

(6) As previously described, (Cunliffe & Shuster, 1969) two papers are used per subject to collect sebum from a 17·5 cm$^2$ area of forehead for a prescribed time (we used a 165 min collection period). (a) Clean forceps are used to remove the papers from each beaker and to return them to their original beakers at the end of the collection. (b) Two beakers per tray are not used and serve as controls.

(7) The tray of beakers is returned to the weighing room and allowed to re-equilibrate to room conditions for at least 2 h.

(8) Post-weights are then measured as described in step 4.

(9) The sebum weight for each subject is determined by subtracting the preweight from the post-weight and adjusting for the control by adding (or subtracting) the average weight lost (or gained) by the two control beakers.

In this study the papers were then processed by the ether extraction technique previously described (Cunliffe & Shuster, 1969). Since we were using this as a second technique, the papers used in our study were prewashed with ether, but this should not be necessary if the direct technique is used alone.

The direct method was compared with the ether extraction method in determining (1) SERs in a group of 43 subjects, (2) left forehead versus right forehead comparisons in a group of 19 subjects and (3) the recovery of sebum from 32 preweighed sebum standards. For the latter, known quantities of sebum were dissolved in a small volume of ether and applied to preweighed papers in beakers. After evaporation of the ether, the beakers were left overnight in the weighing room, then post-weighed, and finally the papers were ether extracted.

To assess the effect of changing humidity on the paper weights, a further experiment was conducted in which a set of five paper-containing beakers were weighed under a range of humidity levels. Finally, the possible effect of corneocyte shedding was evaluated; (1) by direct microscopy of several papers after a $2\frac{3}{4}$ h collection period and (2) by measuring sebum weights via both methods in patients with dry scaling skin due to 13-*cis*-retinoic acid therapy.

## RESULTS

The result for the first group of patients is shown in Fig. 1. Direct weights ranged from 0·31 to 11·60 mg. Corresponding ether extraction weights averaged 90% of the direct values and ranged from 0·38 to 9·90 mg. There was a high degree of correlation between the direct and ether extraction weights ($r = 0{\cdot}991$, $P < 0{\cdot}001$).

Table 1 shows sebum weights from left versus right forehead collections as determined by each of the two methods. The correlations between the left versus right values were high for both techniques (direct method: $r = 0{\cdot}966$, $P < 0{\cdot}001$; ether extraction method: $r = 0{\cdot}963$, $P < 0{\cdot}001$). The greatest percentage variation in left versus right was found with the very low sebum weights (i.e. patients who were prepubertal or were taking 13-*cis*-retinoic acid).

The recovery of sebum from premeasured standards is shown in Figs 1 and 2. In Fig. 1, the direct versus ether extraction results are shown. The ether weights averaged 92% of the direct weights, and there was a good correlation between the two methods ($r = 0{\cdot}997$, $P < 0{\cdot}001$). Figure 2 shows the rate of sebum recovery from the original premeasured weights for each

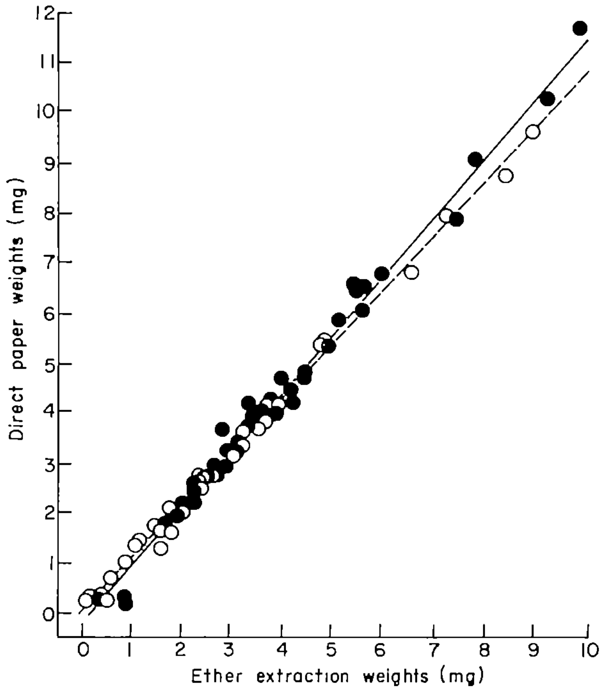

FIGURE 1. Sebum weights determined by direct versus ether extraction techniques. ●——●, Results from 43 normal subjects ($r = 0{\cdot}991$, $P < 0{\cdot}001$). ○– – –○, Results from 32 premeasured sebum standards ($r = 0{\cdot}997$, $P < 0{\cdot}001$).

TABLE 1. Left versus right forehead sebum collections

| | Paper weights (mg) | | Ether extraction weights (mg) | |
|---|---|---|---|---|
| Patient | Left | Right | Left | Right |
| 1 | 1 84 | 1·92 | 1 79 | 2·10 |
| 2 | 1 41 | 1·38 | 1·43 | 1·37 |
| 3 | 2 04 | 2 07 | 2 20 | 1 92 |
| 4 | 3·85 | 4·05 | 3 32 | 3 67 |
| 5 | 2·84 | 2·64 | 2·62 | 2·22 |
| 6 | 0 95 | 0·96 | 0 94 | 0·95 |
| 7 | 1 72 | 2 19 | 1 68 | 2 10 |
| 8 | 0 31 | 0 36 | 0 31 | 0 38 |
| 9 | 1 06 | 1 34 | 1 08 | 1 39 |
| 10 | 2·33 | 2·03 | 2·08 | 1 95 |
| 11 | 0 78 | 0 80 | 0 79 | 0 61 |
| 12 | 2 83 | 3 38 | 2 75 | 3 19 |
| 13 | 2 59 | 2·21 | 2·30 | 2·14 |
| 14 | 2·51 | 1 75 | 2 37 | 1 76 |
| 15 | 2 21 | 2 20 | 1 90 | 1 92 |
| 16* | 0 23 | 0·24 | 0 20 | −0·02 |
| 17† | 0 46 | 0 34 | 0 33 | 0 03 |
| 18† | 0 15 | 0·01 | 0 13 | 0·10 |
| 19† | 0·11 | 0 02 | −0·05 | 0 11 |

* Prepubertal patient.
† Patients on 13-*cis*-retinoic acid.

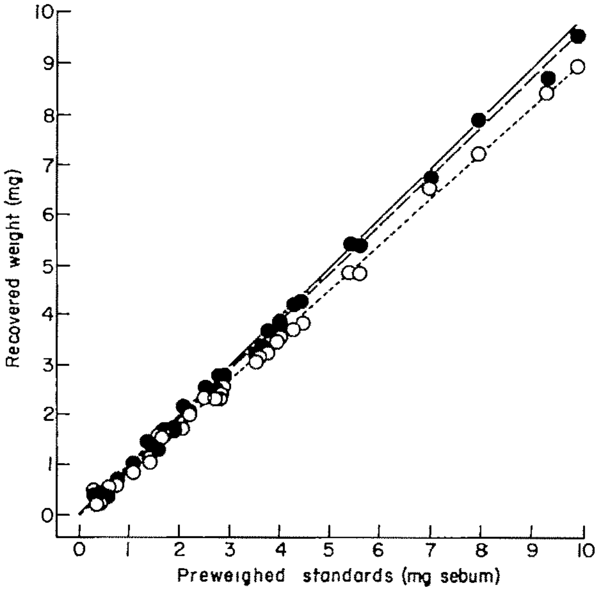

FIGURE 2 Recovery of sebum from premeasured sebum standards. ●– – –●, Direct paper weight method. ○- - -○, Ether extraction method. ———, 100% yield. For both methods $r=0\ 999$ ($P<0\ 001$).

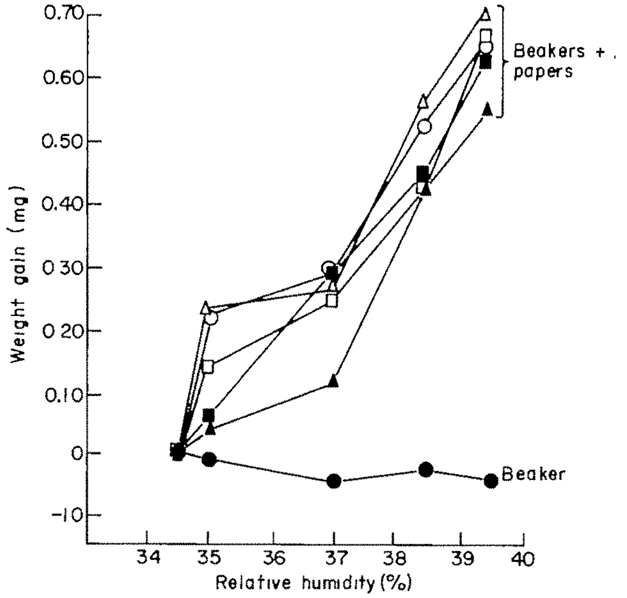

FIGURE 3. Effect of relative humidity on paper weights and on beaker without papers.

technique. The direct method gave weights which showed a good correlation with the original sebum weights ($r = 0{\cdot}999$, $P < 0{\cdot}001$) and recovered 97% of the premeasured weight. The ether extraction method gave weights which showed an equally good correlation with the premeasured weights ($r = 0{\cdot}999$, $P < 0{\cdot}001$), but recovered only 90% of the original sebum.

Figure 3 shows the change in paper weights with change in relative humidity. Over the range of humidity tested, each of the five paper containing beakers gained an average of 0·11 mg per one percent rise in relative humidity. The control beaker, containing no papers, showed no increase in weight with increasing humidity.

Direct microscopy of papers used in a standard $2\frac{3}{4}$ h sebum collection revealed the presence of oil droplets but no detectable stratum corneum cells.

## DISCUSSION

In recent years, several new techniques for collecting and measuring sebum have been introduced including photometric techniques (Saint-Leger *et al.*, 1979; Cunliffe, Kearney & Simpson, 1980) and a method involving collection with bentonite clay from which the sebum is extracted, then measured with quantitative thin layer chromatography (Downing, Stranieri & Strauss, 1982). These methods, however, are not in widespread use and the original quantitative gravimetric method (Strauss & Pochi, 1961), sometimes with modifications (Cunliffe & Shuster, 1969), is still frequently employed.

We have found that it is possible to simplify the gravimetric method by simply pre- and post-weighing the collecting papers, hence eliminating the ether extraction step. Our results demonstrate the accuracy of the direct method as compared with the ether extraction method in measuring sebum collected from patients and from premeasured standards, and in making left versus right forehead collection comparisons.

Our results show that the ether extraction method remains reliable, but it is not without disadvantages. The extraction process involves multiple steps, each of which is a potential source for error. In two of the stages in our laboratory the sebum is transferred (via ether) from one vessel to another. Some loss in weight can be expected with these transfers, and it is probable that this loss is largely responsible for the 8–10% difference in weight between the two techniques as measured from standards and from patients. Another potential source of error in the ether extraction method is contamination of the ether with soluble material which leaves a residue after evaporation. The amount of this residue varies between batches of ether even though the same brand of AnalaR® ether is used. Residue weights range from negligible amounts to up to 0·4 mg per sample (Cunliffe & Shuster, 1969). The controls adjust for this but if contamination is substantial, error is introduced if slightly different volumes of ether are used in extracting the sebum samples.

The direct gravimetric method eliminates these potential problems and has several additional advantages over extraction techniques. It requires less equipment, no ether and less technician time. Hence, it is less expensive and also safer because the fire risk is eliminated. With fewer steps, there are fewer opportunities for error including loss of sebum in processing. This is supported by our measurements of the premeasured sebum standards from which the direct method recovered 97% of the weight while the ether extraction technique recovered only 90%.

The major disadvantage of the direct method is that much more careful attention needs to be given to the weighing process. The cigarette papers change weight with changes in humidity at a rate of about 0·11 mg per two collecting papers for each percentage change in relative humidity. Hence, weighing should only be done under conditions of stable temperature and relative relative humidity. During the weighing process it is necessary for the humidity in the balance chamber to be the same as in the beakers in the room. Hence, a dehydrating agent should not be used in the balance during the weighing period. We wish to emphasize that our balance room is not perfect, but we suspect, like other departments, is probably adequate for the purpose, provided care is taken. In tropical climates, an air conditioned room should be used. Changes in weighing room humidity between the pre- and post-weights are simply adjusted for by the change in control weights.

We considered using containers with air-tight lids so that, once they were capped, the humidity inside the vessel would not change. However, this introduces several more steps and still does not obviate the need for stable humidity conditions at the time of capping the containers for both the pre- and the post-weights.

The other major concern about the direct method is whether it measures more than sebum. Do the papers collect from the skin surface other substances in addition to sebum? The two major contenders are sweat and shedding corneocytes.

Sebum collections are done on subjects who sit quietly in a cool room and are not allowed to drink hot beverages. Under these conditions, sweating does not normally occur. Even if it should, the 2–24 h period between collection and post-weighing would be ample to allow any sweat to evaporate from the papers.

For the stratum corneum shedding, one would expect that the number of corneocytes shed from a 17·5 cm$^2$ area of skin over a 2¾ h period would comprise a negligible weight. However, if this (or other non-sebum factors) were to contribute to the paper weights, the regression line comparing the paper and extraction weights would cross the positive $y$ axis, which it does not (Fig. 1). Further support for a negligible corneocyte factor comes from our observations that, firstly, with direct microscopy no corneocytes could be seen on the collecting papers used for a 2¾ h collection and secondly, the paper weights from the dry scaling skin of three patients taking 13-*cis*-retinoic acid was not appreciably higher than the ether extraction weights.

The results from the 13-*cis*-retinoic acid patients, however, do point to a problem in sebum measurements which is common to both techniques. As can been seen from Fig. 1 and Table 1, when very low levels of sebum are collected (a situtation which pertains mainly to prepubertal subjects and patients taking sebosuppressive drugs) the inherent errors in both techniques are magnified. However, even for these low values, the direct method performs at least as well as the ether extraction technique.

We conclude that the direct gravimetric method provides a simple, accurate and efficient technique for measuring sebum, and it is less expensive than methods currently in use. Our data suggests that non-sebum factors such as sweat or shedding corneocytes do not play a role. It appears that the direct method measures only sebum, and at a recovery rate higher than that for the ether extraction technique.

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# Reduction in murine Langerhans cell ATPase staining following topical but not systemic treatment with steroid and non-steroid immunosuppressants

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## SUMMARY

The effects on murine Langerhans cells (LC) of steroid and non-steroid immunosuppressive drugs which are commonly used for long-term immunotherapy of human patients were investigated. Hydrocortisone, prednisolone, cyclosporin A or azathioprine was administered daily for 7 consecutive days either topically by application to the skin, or systemically by intraperitoneal injection. LC densities were determined on the day following cessation of treatment by staining for the plasma membrane-bound enzyme adenosine triphosphatase (ATPase). All immunosuppressants caused a significant reduction in ATPase-positive LC when administered topically, but not systemically. The systemically administered drugs, although given in high concentration, may not have penetrated the epidermis in sufficient concentrations to disrupt the LC membrane. These observations are consistent with long term immunosuppressants depleting cutaneous LC by bone marrow suppression rather than by a direct effect on LC.

Langerhans cells (LC) are dendritic cells found in the mammalian epidermis where they function as a cutaneous antigen-presenting cell, thus having a central role in local immunological defence (Halliday & Muller, 1984). Patients who have received renal transplants have an increased incidence of pre-malignant and malignant skin lesions (Blohmé & Larkö, 1984), as well as cutaneous fungal and viral infections (Boyle *et al.*, 1984). Since renal allograft recipients have been found to have a decreased number of cutaneous LC (Sontheimer *et al.*, 1984), we have investigated the effect of maintenance immunosuppressive agents on cutaneous LC. Although the effects of some topically applied steroids on LC density have been investigated (Lynch, Gurish & Daynes, 1981; Norlund, Ackles & Lerner, 1981, Belsito *et al.*, 1982; Berman *et al.*, 1983; Aberer *et al.*, 1984), the agents commonly used for immunosuppressive therapy in

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humans have not been studied. The present paper describes the effects of azathioprine, prednisolone, hydrocortisone and cyclosporin A applied either systemically or topically, on the number of adenosine triphosphatase-positive LC in mouse epidermis.

## METHODS

### *Animals*

Male C57BL mice obtained from the central Animal House, University of Tasmania, were used with approval from the Ethics Committee.

### *Immunosuppressant administration*

Mice receiving topical immunosuppressants were painted daily on the shaved dorsal trunk skin with approximately 0·1 ml of the following drugs for 7 consecutive days (days 0–6): cyclosporin A (CsA) (Sandoz Ltd., Switzerland), 5 mg/ml in lanoline (F.H.Faulding and Co., Australia); hydrocortisone acetate ointment (HC) (E.R.Squibb and Sons, Australia) 10 mg/ml; methylprednisolone sodium succinate (PD) (Upjohn, Belgium) 20 mg/ml in lanoline; azathioprine (AZ) (Wellcome, U.K.) 10 mg/ml in lanoline. Control mice were treated with lanoline alone.

Mice receiving systemic immunosuppressants were given daily intraperitoneal injections (0·05 ml) of the following drugs for 7 consecutive days (days 0–6): CsA, 0·5 mg/mouse/day dissolved in ethanol:saline (1:1, v/v); hydrocortisone sodium succinate (HC) (Glaxo, U.K.), the equivalent of 2 mg HC/mouse/day dissolved in distilled water; PD, 2 mg/mouse/day dissolved in saline; AZ, 1 mg/mouse/day dissolved in saline. Control mice received solvent alone, either ethanol:saline, distilled water, or saline. As no difference was found between these solvent treatments, the results are shown as one solvent treated group.

### *Preparation of skin samples for LC enumeration*

On the day following the final administration of the immunosuppressant drug (day 7), the mice were killed by anaethesia and cardiac puncture. The dorsal trunk skin was shaved, depilated (using Veet®, Reckitt and Colman Ltd., U.K.), rinsed of depilant with tap water, and surgically removed. The skin samples from the topically immunosuppressed mice were taken from the drug-treated area. Epidermal sheets were prepared from the skin by a modification of the method of Baker & Habowsky (1983). The skin was divided into 5 mm squares using a perspex template, subcutaneous fat was removed, and the samples were incubated overnight at room temperature in phosphate-buffered saline (PBS, pH 7·3) containing 20 mM ethylenediamine-tetra-acetic acid (EDTA) and 0·001% trypsin (Boehringer, W. Germany). The keratin and dermal layers were gently removed from the epidermis using fine forceps and a dissecting microscope, while the sample was submerged in PBS containing 1 mM $MgCl_2$ ($PBS/Mg^{2+}$).

### *ATPase staining of epidermal LC*

LC were identified in epidermal sheets by staining for the enzyme adenosine triphosphatase (ATPase) using a method previously described (Muller, Halliday & Knight, 1985).

### *LC enumeration*

For each mouse the number of LC in the dorsal trunk skin was enumerated microscopically in four replicate epidermal sheets by two independent observers; the total area assessed per mouse was 2·5 mm². Following LC counting, the area of each sheet was measured using a Bioquant II basic measuring program, volume 185 (R & M Biometrics, U.S.A.), for an Apple IIe computer.

The percentage increase in epidermal sheet size was used to correct for stretching which occurred during preparation of epidermal sheets.

### *Blood leukocyte quantitation*

Blood was collected by cardiac puncture into heparin (20 units/ml, Weddel Pharmaceuticals Ltd., U.K.). Blood leukocytes (WBC) were enumerated using a Coulter Counter (Coulter Electronics, U.K.).

### *Statistical analysis*

LC densities and WBC counts from the different groups of mice were compared using an unpaired Wilcoxon rank sum test.

## RESULTS

### *Topical immunosuppression*

Topical application of all four immunosuppressants resulted in a significant decrease in the mean number of ATPase-positive LC in each test group compared with the solvent-treated control group (Table 1). Topically applied CsA decreased the ATPase-positive LC by 35%, HC caused a 42% reduction, PD a 35% reduction and AZ a 51% reduction, each drug inducing a similar decrease in LC numbers, with no significant differences between the groups. The LC densities from individual mice are shown in Fig. 1. A considerable range of LC densities was observed in the control mice; however, all four immunosuppressants reduced the LC densities from most treated mice to below that found in any of the controls. In some drug-treated mice the LC density was less than half that found in any control mouse.

The mean numbers of circulating WBC were unaffected by topical application of CsA, HC or PD, but were significantly reduced by AZ (Table 1).

### *Systemic immunosuppression*

In contrast to the topically applied immunosuppressants, the same four drugs applied systemically did not have any significant effect on the mean number of ATPase-positive LC

TABLE 1. Effects of topical immunosuppression[a] on epidermal Langerhans cell (LC) and blood leukocytes (WBC)

| | Langerhans cells per mm$^2$ | | Blood leukocytes per ml × 10$^6$ | | |
|---|---|---|---|---|---|
| | Mean | $P^b$ | Mean | $P^b$ | Number of mice |
| Control | 362 | | 2 647 | | 12 |
| Cyclosporin A | 234 | $<0{\cdot}05$ | 3·827 | n.s. | 7 |
| Hydrocortisone | 209 | $<0{\cdot}01$ | 2 024 | n.s. | 7 |
| Prednisolone | 234 | $<0{\cdot}05$ | 1·604 | n.s. | 8 |
| Azathioprine | 177 | $<0{\cdot}01$ | 1 134 | $<0{\cdot}05$ | 8 |

[a] Drugs applied for 7 consecutive days (days 0–6), LC and WBC measured on day 7.

[b] Statistical comparison with control (Wilcoxon rank sum test).

n.s., not significant.

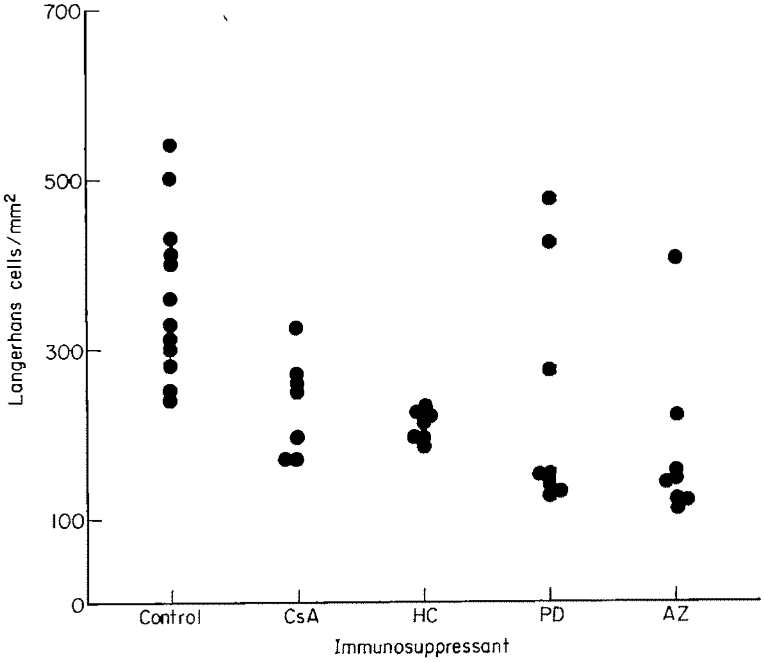

FIGURE 1. Effects of topical immunosuppression with cyclosporin A (CsA), hydrocortisone (HC), prednisolone (PD) or azathioprine (AZ) on Langerhans cell density as determined by ATPase staining.

(Table 2). The LC densities from individual mice are shown in Fig. 2. The range of values obtained for each drug-treated group falls within the range obtained for the solvent-treated control group.

The mean numbers of circulating WBC found in the groups of mice systemically immunosuppressed with CsA, HC and AZ were significantly reduced compared with the control group (Table 2). Systemic PD, however, did not significantly reduce the WBC count.

TABLE 2. Effects of systemic immunosuppression[a] on epidermal Langerhans cells (LC) and blood leukocytes (WBC)

| | Langerhans cells per mm$^2$ | | Blood leukocytes per ml × $10^6$ | | |
|---|---|---|---|---|---|
| | Mean | P[b] | Mean | P[b] | Number of mice |
| Control | 385 | | 3.356 | | 14 |
| Cyclosporin A | 329 | n.s. | 2 325 | <0 05 | 6 |
| Hydrocortisone | 312 | n.s. | 1 655 | <0 05 | 7 |
| Prednisolone | 401 | n.s. | 2 509 | n.s. | 6 |
| Azathioprine | 407 | n.s. | 1 739 | <0 005 | 8 |

[a] Drugs applied for 7 consecutive days (days 0–6), LC and WBC assessed on day 7.
[b] Statistical comparison with control (Wilcoxon rank sum test).
n.s., not significant.

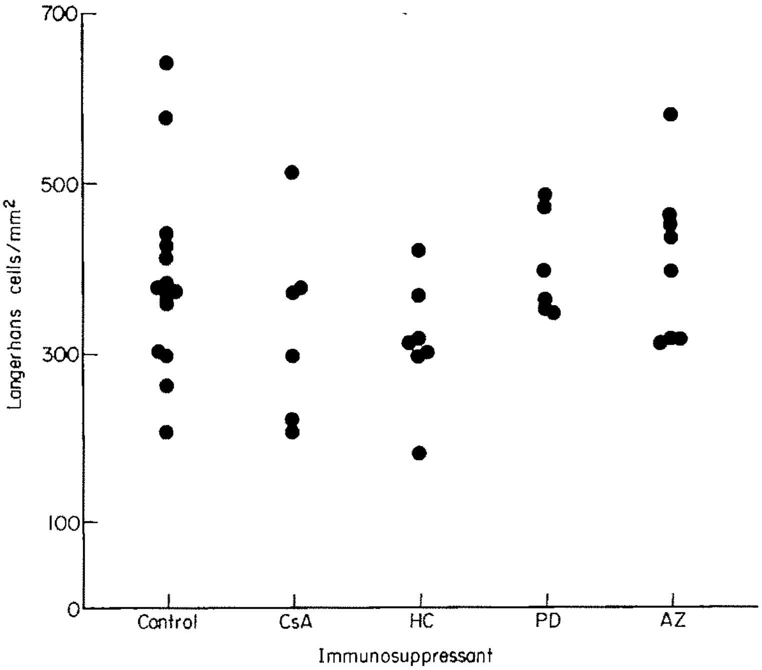

FIGURE 2. Effects of systemic immunosuppression with cyclosporin A (CsA), hydrocortisone (HC), prednisolone (PD) or azathioprine (AZ) on Langerhans cell density as determined by ATPase staining.

## DISCUSSION

This study demonstrates that the topical application of cyclosporin A, hydrocortisone, prednisolone or azathioprine causes a substantial reduction in the number of cutaneous ATPase-positive LC. In contrast when applied systemically these same drugs had no such effect.

Previous studies have also found topical administration of steroids to cause a reduction in some LC markers. For example, topical application of triamcinolone acetonide (TAC) or betamethasone dipropionate has beeen reported to decrease the markers Fc, C3b and Ia, but not T6 from epidermal LC; these steroids therefore disrupted the LC membrane without depleting the cells from the epidermis (Berman *et al.*, 1983). Topical application of the steroids betamethasone dipropionate or fluocinonide have also been shown to inhibit ATPase activity on the surface of LC, as well as contact sensitivity to dinitrofluorobenzene (Lynch *et al.*, 1981). Our experiments extend these results by demonstrating that topical, but not systemic administration of cyclosporin A or azathioprine, as well as the steroids prednisolone and hydrocortisone, depresses the ATPase activity of the LC plasma membrane.

Both topical and systemic administration of TAC have previously been found to deplete mouse skin of Ia-positive LC, requiring 7 weeks for the LC to return to control values (Norlund *et al.*, 1981; Aberer *et al.*, 1984). This long recovery period suggests that systemic TAC, rather than affecting Ia on the LC surface, most probably depletes LC from the epidermis. TAC therefore appears to have a more potent effect on LC than the more routinely used drugs which we assessed.

Belsito *et al.* (1982) found that systemic application of prednisolone decreased the ATPase activity of guinea-pig LC, requiring 7 days to return to control levels. This short recovery time suggests that the LC were not depleted, but had lost their surface markers. In the present experiments on mice, systemic prednisolone had no such effect, possibly because our staining procedure for ATPase detected lower levels of ATPase surviving the steroid treatment, or because of a species difference.

Our finding that topical, but not systemic, immunosuppressants have a direct effect on LC may be due to the drugs reaching the epidermis in higher concentrations when administered topically than systemically. The drugs were administered systemically in very high doses, causing death of a small number of animals. However, it may be that the drugs failed to enter the epidermis from the blood stream at appropriate concentrations or in an active form. While topical immunosuppression reduced the number of LC in most mice to below that found in any control mouse, a small number of treated mice maintained a relatively high LC density, this variability possibly being related to the large normal range.

The present observations that topical hydrocortisone and prednisolone deplete LC markers is in agreement with other reports using different steroids (Berman *et al.*, 1983; Lynch *et al.*, 1981). As azathioprine reduced blood monocyte and lymphocyte numbers (Rees & Lockwood, 1982) it is not surprising that it affects LC. However, the decrease in LC ATPase induced by topical cyclosporin A is unexpected as this drug is not cytotoxic and has no anti-inflammatory action (Bendtzen, Tuede & Svenson, 1984), but specifically inhibits the production of lymphokines by T-inducer cells (Bunjes, 1983; Lafferty, Borel & Hodgkin, 1983).

The clinical implication of this study is that systemically administered immunosuppressants most probably do not directly affect cutaneous LC. These cells only proliferate very slowly in the skin (Mackenzie, 1975; Gschnait & Brenner, 1979), and, as a stable cell population derived from bone-marrow precursors (Stingl, Tamaki & Katz, 1980), only long-term systemic drug treatment which depresses bone marrow function would be expected to cause a substantial reduction in LC numbers. Long-term immunosuppression-induced depletion of LC may then allow cutaneous tumour cells and fungal and viral infections to evade local immunological defence mechanisms.

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# A new method for studying epidermalization *in vitro*

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## SUMMARY

A new method for studying epidermalization *in vitro* is described. It consists of inserting a punch biopsy that serves as a source of epidermis into dermal equivalent freshly made up, with fibroblasts mixed in a collagen matrix. Fibroblasts cling to collagen fibrils and contract the matrix, leading in 3 days to a resistant dermal equivalent holding the punch biopsy firmly in place. At day 5, a culture medium favouring epidermal growth was used and a fringe of a new epidermis appeared around the punch, the area of which grew linearly with time. This new epidermis showed a pattern of differentiation similar to epidermis *in vivo*, with cuboidal basal cells, keratohyalin granules, membrane coating granules and the expression of the 65–67 kd keratin subset.

The method seems to combine the advantages of the explant technique and of classical keratinocyte cultures, providing the researcher with a large quantity of differentiated epidermis, the pharmacologist with simple and quantitative system in which to study modifications of growth and differentiation of epidermis, and the plastic surgeon with a possible material for skin grafting.

In spite of considerable efforts and recent improvements, a qualitative and quantitative method for studying epidermalization in conditions resembling those *in vivo* is still lacking.

Epidermal differentiation *in vitro* was greatly improved by using a feeder layer of fibroblasts (Rheinwald & Green, 1975) or the macromolecules of the matrix (Woodley *et al.*, 1980; Lillie, MacCallum & Jepsen, 1980). These discoveries underline the key role of the dermis in epidermal differentiation. Thus, it seemed logical to use the dermal equivalent previously described, as a physiological substrate for epidermal growth (Bell, Ivarsson & Merrill, 1979; Bell *et al.*, 1981b). However, the differentiation of the epidermis obtained from dissociated keratinocytes is not really better than that obtained by other methods (Bell *et al.*, 1983).

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The explant culture has been used frequently because it gives a good model of adult epidermal growth and differentiation (Halprin, Lueder & Fusening, 1979; Van der Schueren, Cassiman & Van den Berghe, 1979). This method yields differentiated epidermis, but gives a very small quantity of biological material and does not allow analysis of the role of the dermal components. Furthermore, this technique allows only short-term experiments.

Inserting a small punch biopsy as a source of epidermal cells, in a dermal equivalent as a physiological substrate, we have obtained a circular growth of the epidermis around the implanted biopsy. This new epidermis was easy to distinguish and to separate from the epidermial cells in the original biospy. Its growth was easy to quantify by planimetry and measurement of DNA content. It can be obtained in very large quantities. Its differentiation was close to that observed *in vivo,* including the formation of keratohyalin granules, the expression of 65–67 kd keratins and even the appearance of numerous membrane coating granules. Thus, this method seems to be of interest for studying epidermalization.

## METHODS

### *Fibroblast cultures*

Fibroblast cultures were established from 1 or 2 mm diameter punch biopsies of human skin obtained from normal volunteers or from breast plastic surgery. The cells were propagated in Earle's Modified Eagle culture Medium (EMEM, Flow Labs) containing 10% of human AB serum, 100 IU/ml penicillin, 100 μg/ml streptomycin (Flow Labs) and 2·5 μg/ml amphotericin B (Squibb). The tissue culture flasks were kept at 37°C in a 5% $CO_2$ atmosphere.

### *Dermal equivalent*

Fibroblasts from subconfluent or just confluent cultures were removed by trypsin treatment (0·05% trypsin 1:250 and 0·02% EDTA; Flow Laboratories). Cells were washed with culture medium and suspended at $4 \times 10^5$ cells/ml in complete medium. The dermal equivalents were prepared in 60 mm bacteriological dishes as previously described (Bell *et al.*, 1979). Each dish contained 4·6 ml of concentrated EMEM with antibotics, 0·9 ml of human AB serum, 3 0 ml of collagen solution (2 mg/ml in 1:1000 acetic acid solution; extracted from rat tail tendons), 0·5 ml of 0·1 M NaOH and 1·0 ml of fibroblast suspension. Dishes were placed at 37°C in a 5% $CO_2$ atmosphere. A gel formed immediately after the mixture was made.

### *Epidermalization*

Three-millimeter punch biopsies, without hypodermal fat, were taken either from the arm of normal volunteers after a local anaesthesia with 1% xylocaine without adrenaline, or from discarded skin after breast plastic surgery. After several rinses in culture medium with antibiotics, each biopsy was implanted at the centre of a freshly cast collagen gel containing fibroblasts from the same or another donor. The implantation was made under a stereomicroscope so that the dermo-epidermal junction of the biopsy could be positioned level with the surface of the gel.

As a result of interactions between fibroblasts and self-assembled collagen fibrils, the volume of the gel was reduced twenty-fold or more in the course of several days (Bell *et al.*, 1979). During this process, the implanted biopsies became tightly held in place. By 5 days the contracted gel resembled a tissue. At this time, the skin equivalents were positioned on stainless steel grids that kept them elevated in the Petri dishes. The culture medium was replaced with fresh EMEM plus antibiotics and 10% human AB serum, added with 20 ng/ml of EGF

(Collaborative Research), 0·4 μg/ml of hydrocortisone (Roussel) and cholera-toxin at a final concentration of $10^{-10}$ M (Sigma). Enough medium was used to allow the liquid to just cover the surface of the skin equivalent.

Large dermal equivalents were also prepared in dishes of 25 cm diameter, using the same ingredients as described previously. The final volumes were 125 or 500 ml according to the thickness desired. Rat tail collagen, however, was replaced by calf skin collagen at the same concentration in acetic acid 1 : 1000. This collagen preparation has been used to make collagen compresses for human surgery (Pangen: Fournier-Dijon) and can be obtained in large quantities (SADUC). These dermal equivalents were implanted with forty 2-mm diameter punch biopsies.

### *Histology*

Light and electron microscopy were performed on 10-day-old material. Fixation with Karnovsky's medium, dehydratation, embedding in Epon and sectioning were performed as previously described. Sections 0·5 μm thick were stained for light microscopy with acid Giemsa (Dubertret *et al.*, 1980).

### *Keratin extraction and identification.*

The epidermis that had grown out from a punch biopsy was gently removed from the dermal equivalent, with forceps, 15 days after the insertion of the biospy. The area of the punch itself was cut out and discarded.

As a control, fresh human epidermis was obtained by the suction blister method from the skin of normal volunteers (Lowe & Van der Leun, 1968).

The epidermal sheets so obtained were homogenized in 10 mM Tris-HCl (pH 8) containing 0·5% Triton X100, 1·5 M KCl, 10 mM NaCl, 2 mM dithioerythritol (Sigma). After centrifugation at 10,000 ***g*** for 5 min at 4°C, the water-insoluble pellet was suspended in 10 mM Tris HCl (pH 8) containing 5% sodium dodecyl sulphate (SDS) and 5% 2-mercaptoethanol, and heated for 1 min at 100°C. Samples were electrophoresed on 10% polyacrylamide slab gels in the presence of SDS (Laemmli, 1970). Polypeptides were stained in the gel with Coomassie Blue and their molecular weights determined from the migration of standard proteins run in the same gel; these were rabbit phosphorylase (92·5 kd), bovine serum albumine (67 kd), bovine catalase (57·5 kd), ovalbumin (43 kd), pig lactate dehydrogenase (36 kd), carbonic anhydrase (29 kd), soyabean trypsin inhibitor (20 kd) and bovine lactalbumin (14·4 kd).

For immunoblotting experiments, the polypeptides were transferred electrophoretically (65 V for 2 h at 4°C) from unstained gels onto nitrocellulose sheets (BA 85; Schleicher and Schull) under the conditions described by Towbin, Staehelin & Gordon (1979). The sheets were incubated with antikeratin guinea-pig antibodies, prepared with purified bovine hoof prekeratin and staining all keratin subsets (Miles). After washing, the sheets were incubated with anti-guinea-pig IgG rabbit immunoglobulins conjugated with horseradish peroxidase (Cappel Laboratories) and the peroxidase visualized using diaminobenzidine chlorhydrate under conditions described previously (Roseto *et al.*, 1984).

### *Planimetric measurement*

To visualize the epidermal outgrowth over the surface of the dermal equivalents, Nile Blue sulphate (Sigma) was added to the culture medium at a final concentration of 1 : 10,000. After 30 min incubation at 37°C, the cultures were rinsed for 15 min in a 9% NaCl solution. The area of the epidermal outgrowth was measured with a Nachet NS 1000 image analyser on photographs taken at × 0·96 magnification.

### *Measurement of DNA content*

After Nile Blue sulphate staining, the skin biopsy was cut out with a punch of appropriate diameter. The skin equivalent was then incubated for 30 min in serum-free medium with 1·2 μg/ml of Dispase (Boeringher). After several rinses in PBS the epidermis was removed from the dermal equivalent and hydrolysed in 50 μl of 2% Triton X100 and 450 μl of 1N $NH_4OH$ for 1 h at 100°C. DNA content was assayed using a fluorometric procedure (Fiszer-Szafarz, Szafarz & De Murillo, 1981). Purified fetal calf thymus DNA (Sigma) was used as a standard and hydrolysed Nile Blue sulphate solution as a control.

## RESULTS

### *Outgrowth of epidermis from the punch biopsy*

By 5 days, when the skin equivalent was transferred onto a grid, a narrow fringe of epidermis had appeared around the punch biopsy. The area of the newly grown epidermis increased linearly with time (Fig. 1A–C). Epidermal DNA content was a linear function of the area of the outgrowth after 9 days in culture (slope ± s.e. of the slope: 0·23 ± 0·02 μg/mm$^2$).

Histologically, by 10 days, the punch biopsy was held in the dermal equivalent, even if a light epidermal epiboly occurred around the explant before reaching the dermal equivalent (Fig. 2). On the dermal equivalent itself, the basal keratinocytes of the growing epidermis were well organized into a coherent layer of cuboidal cells. From this basal layer, a multilayered epidermis differentiated. In the stratum spinosum the desmosomes were as numerous as in normal skin *in vivo* (Figs 2–4). Higher in the epidermis, the keratinocytes were flat, with no visible nuclei, forming a thick and compact cornified layer that desquamated (Figs 2–4). A thin granular layer

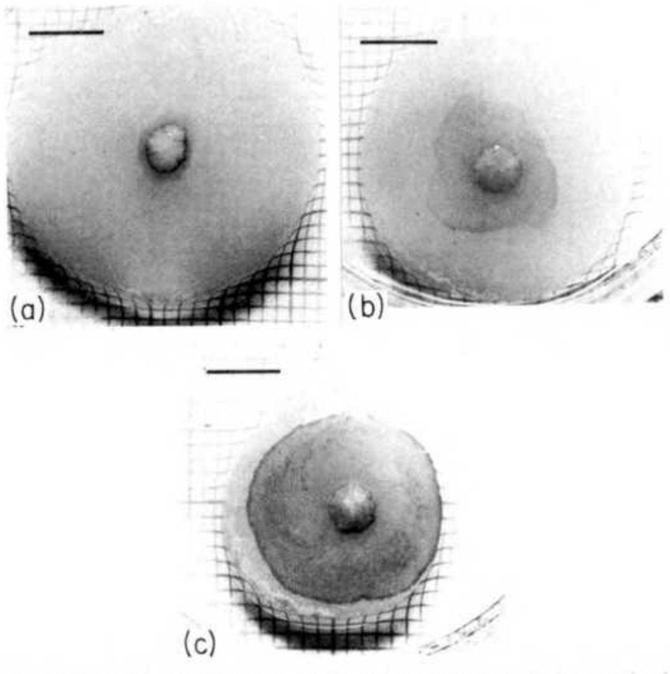

FIGURE 1. Epidermal outgrowth on the surface of the living dermal equivalent, stained with Nile Blue sulphate, at 5 days (A), 10 days (B) and 15 days (C). Bar = 1 cm.

with some typical keratohyalin granules was observed, as well as many membrane coating granules immediately under the stratum corneum in which the cells were characterized by a thick cornified envelope (Fig. 4). In spite of the close junction observed between the newly-formed epidermis and the dermal equivalent as observed with the light microscope, a lamina densa and hemidesmosomes could not be found with the electron microscope after 10 days. In sections approaching the edges of the newly formed epidermis, the epidermal layers became less numerous and the stratum corneum gradually disappeared.

### *Expression of keratins in the epidermal outgrowth on the dermal equivalent*

In the normal human epidermis we found the 67–65, 58, 56·5 and 50 kd keratins (Fig. 5 lanes A and C). In the growing epidermis covering the dermal equivalent the major keratin species

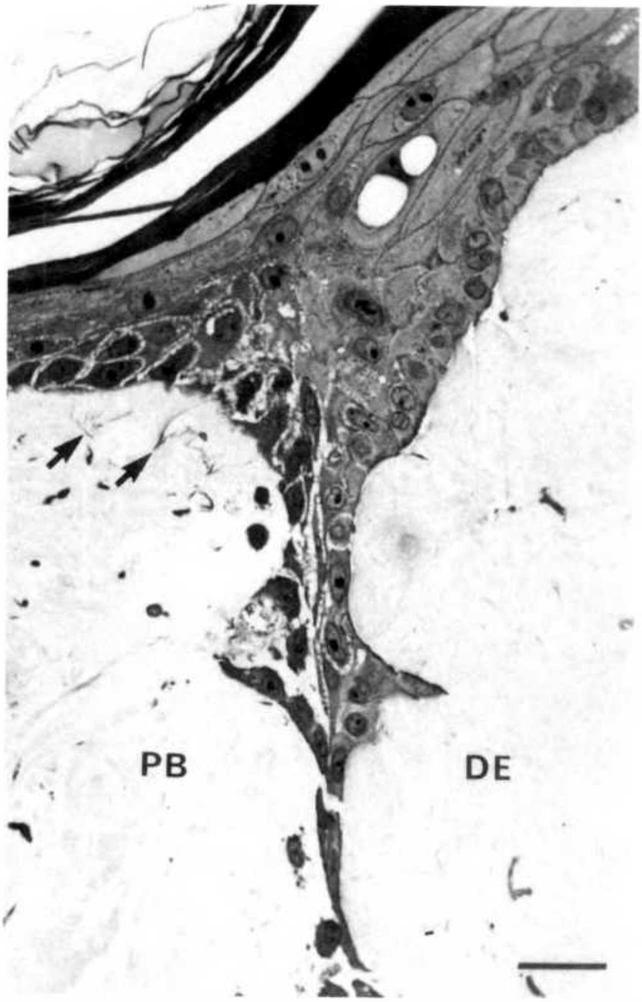

FIGURE 2. Semi-thin section, at 10 days, at the junction of the punch biospy (PB) and the dermal equivalent (DE). On the punch biospy, keratinocytes have degenerated, the dermo-epidermal junction is very loose in spite of the presence of anchoring fibrils (→). The outgrowth of keratinocytes reaches the surface of the dermal equivalent and differentiates into a multilayered epidermis. Bar = 100 μm.

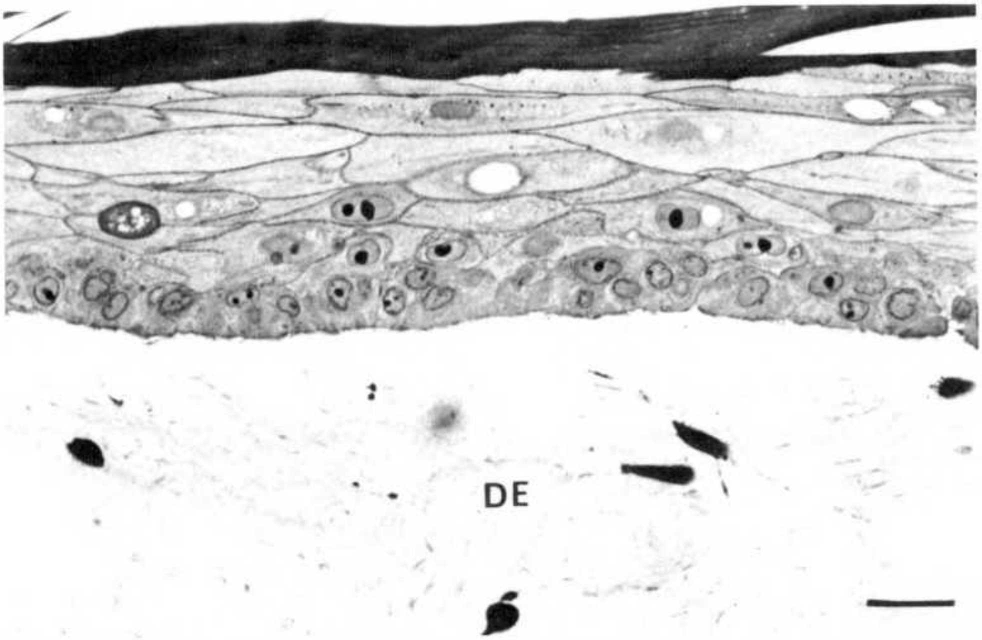

FIGURE 3. Semi-thin section, at 10 days, on living dermal equivalent (DE). Basal keratinocytes are organized into a layer of cuboidal cells. The different strata are well attached. In the upper layers, cells become flat, lose their nucleus and form a compact cornfied layer that desquamates. Bar = 100 μm.

usually expressed in keratinocyte cultures were present: mainly 48, 50, 56·5 and 58 kd. In addition, the 65–67 kd keratin subset, typical of *in vivo* terminal differentiation, was also expressed, but in a somewhat smaller amount than *in vivo* (Fig. 5 lanes B and D).

### *Production of a large area of skin equivalent*

The 25 cm diameter collagen gel, populated with forty 2 mm punch biopsies (125 mm$^2$ of skin) contracted to a size of 8·5 cm diameter (6000 mm$^2$). At equilibrium, the biopsies were situated 1 cm apart. After 12 days, when epidermalization was sufficient for utilization for grafting (Fig. 6) the initial epidermal area had been multiplied by a factor of 50.

## DISCUSSION

When the dermal equivalent, constituted *in vitro* with human skin fibroblasts and collagen, is used as a substrate for epidermis growing out from punch biopsies, the quality of the epidermis is superior to that obtained when a suspension of keratinocytes is applied to the dermal equivalent (Bell *et al.*, 1983). To the best of our knowledge the reconstitution *in vitro* of human differentiated epidermis is rarely so complete (Holbrooks & Hennings, 1983). In our technique keratinocytes synthesized high molecular weight keratins including the 65–67 kd proteins that are generally absent in cell cultures (Sun *et al.*, 1983; Eichner, Bonitz & Sun, 1984), except when cells are grown in the presence of delipidized serum (Fuchs & Green, 1981). However, the keratin pattern obtained in our experiments was somewhat different from the one observed in normal human epidermis, but similar to the one expressed in hyperproliferative epidermal disorders *in vivo* (Weiss, Eichner & Sun, 1984): the 65–67 kd keratins seemed to be less

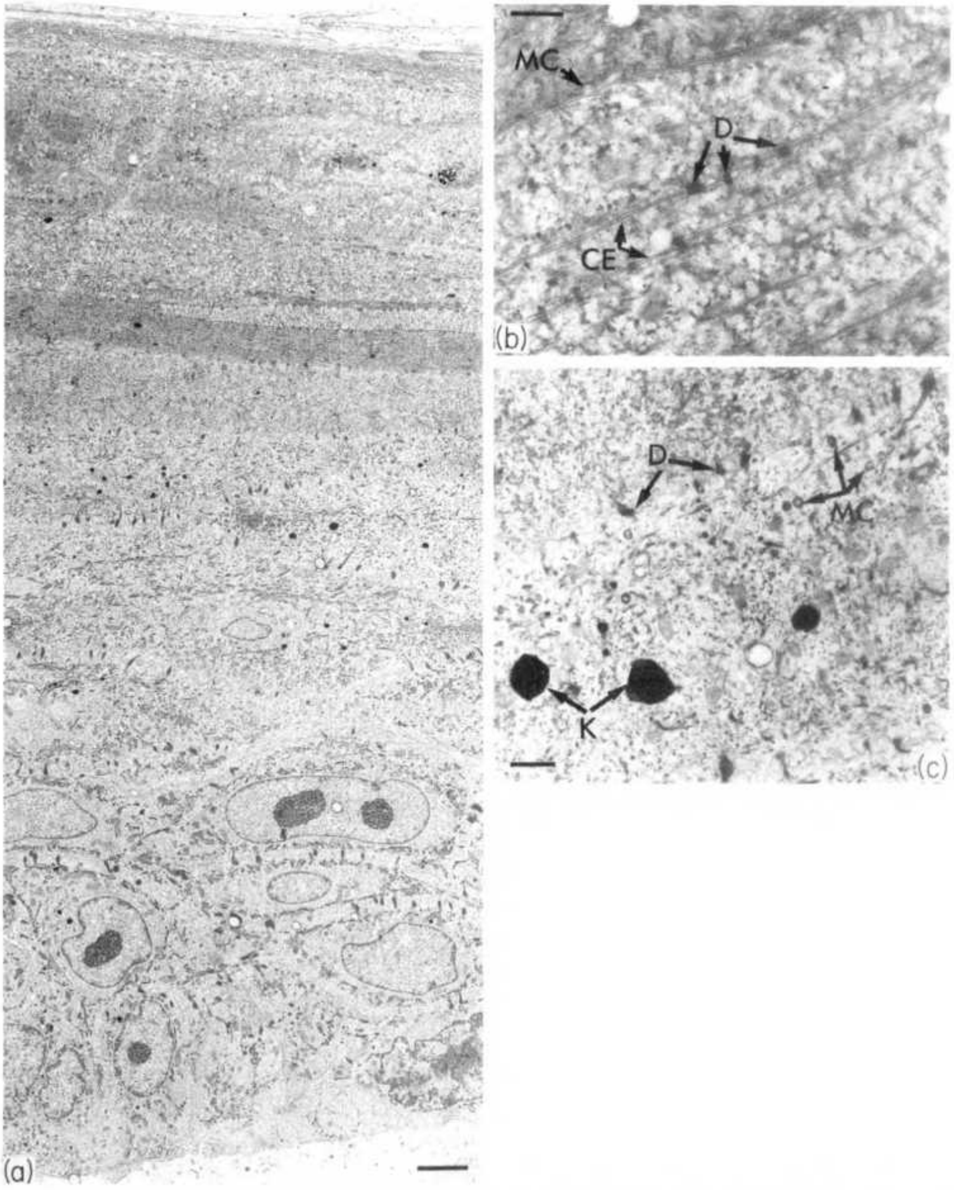

FIGURE 4. Electron microscopy, at 10 days, of epidermis from a biopsy overgrowing the living dermal equivalent: general view (a) and details of the upper layers (b and c). Basal keratinocytes are cuboidal. In the stratum spinosum the frequency of desmosomes (D) is similar to that *in vivo*. As they differentiate, cells become flat and lose their nuclei. Keratohyalin granules (K) and membrane coating granules (MC) are present in the upper layers, and a thick cornified envelope appears (CE). There is good cohesion between the different layers. (a) Bar = 25 $\mu$m (b and c) Bar = 5 $\mu$m.

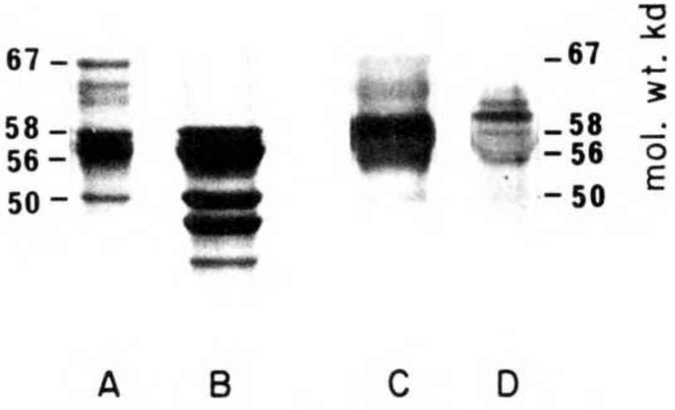

FIGURE 5. Polyacrylamide gel electrophoresis stained with Coomassie Blue (lanes A and B), and immunoblot reacted with antiserum staining all keratin subsets and revealed by diaminobenzidine reaction (lanes C and D). Normal *in vivo* epidermis (A and C) and *in vitro* outgrown epidermis (B and D). *In vitro* epidermis expresses the keratin species usually found in keratinocyte cultures, but in addition the 65–67 kd keratin subset is present, as *in vivo*.

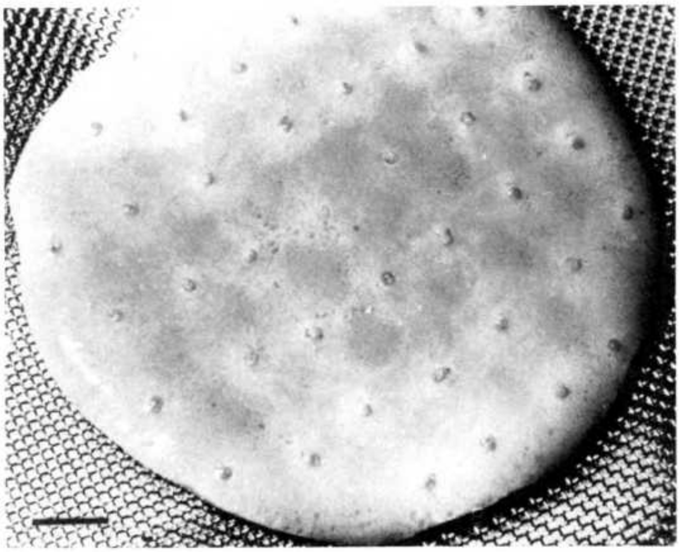

FIGURE 6. Living skin eqivalent at 12 days, on a stainless steel grid. The collagen gel of 25 cm diameter was implanted with punch biospies every 4 cm. After contraction by fibroblasts, the dermal equivalent is 8·5 cm in diameter with biopsies separated from each other by 1 cm. The outgrowth of epidermal cells from the biopsies nearly covers the entire dermis. Bar = 1 cm.

abundant in the model system than in normal epidermis and keratins of molecular weight lower than 50 kd were present.

Our histological and ultrastructural observations confirm the quality of the differentiation obtained. In particular, to the best of our knowledge, the appearence of membrane coating granules observed in our model had never been found in cultures of human epidermis until now (Holbrook & Hennings, 1983), but only in cultures of rat epithelial cells when they grew on collagen gels (Lille *et al.*, 1980).

The absence of basement membrane in skin equivalents made with human cells is not surprising since its formation requires an *in vitro* growth period of at least 1 or 2 months (Hirone & Taniguchi, 1979). With rat cells, a basement membrane is seen in the skin equivalent in 15 days and is always present by 7 days after grafting onto a host *in vivo* (Bell *et al.*, 1983).

The quality of the epidermal differentiation observed can be attributed to two factors, the growth on a collagenous matrix containing living fibroblasts, and the use of a punch biopsy as a source of keratinocytes.

Collagen alone is recognized as a good substrate for keratinocytes (Holbrook & Hennings, 1983). Collagen improves cellular attachment and plating efficiency and seems essential for obtaining basal lamina formation *in vitro* (Hirone & Taniguchi, 1979; Lillie *et al.*, 1980). When living cells are added to the collagen matrix they modify it physically (Bell *et al.*, 1979) and chemically (Nusgens *et al.*, 1984) giving it important properties characteristic of the dermis. A lethally irradiated feeder layer of fibroblasts (Reinwald & Green, 1975) or lethally irradiated skin (Woodley *et al.*, 1980) promote subcultures of keratinocytes and extend the lifetime of the culture, but the system remains very different from that *in vivo*. The importance for normal epidermal growth of vital dermis and the requirement for diffusible dermal products have already been suggested (Mackenzie & Fusenig, 1983). In the dermal equivalent, fibroblasts surrounded by collagen exhibit a differentiation like that *in vivo* and are biosynthetically active (Nusgens *et al.*, 1984; Coulomb *et al.*, 1983, 1984). Thus, they could contribute to the excellent differentiation of the epidermis.

The keratinocytes that escape from the well constituted epidermis of the biopsy form a new epidermis as they do during normal cicatrisation (Halprin *et al.*, 1979; Van der Schueren *et al.*, 1979). This directional outgrowth from a normal epidermal source seems to promote the formation of a cuboidal germinative layer of cells usually not seen *in vitro* after seeding trypsinized keratinocytes. This suggests that the shape of the cells of the germinative layer could be important for the expression of a normal sequence of differentiation.

The method described provides the researcher with a large quantity of differentiated epidermis and the pharmacologist with an easy and quantitative system for studying modification of growth and differentiation of epidermis.

The possibility of producing large quantities of living skin equivalent by the punch biopsy method could be also of interest for clinical applications. We have already shown that grafting onto rats is possible (Bell *et al.*, 1981a, 1981b; Hull *et al.*, 1983). In addition, dermal equivalent allografts are immunologically acceptable in the rat model (Sher *et al.*, 1983). We have also found that the dermal equivalent can be stored at physiological temperature in an incubator for more than a year, or in a frozen state (E.Bell, unpublished results). Hence, the delay in producing large amounts of skin equivalent can be greatly reduced. If 1 mm punch biopsies are implanted in previously prepared dermal equivalent, our preliminary results suggest that only 0·5% of the skin could be sufficient for the reconstruction of the whole skin of the body, in 2 weeks. The results of the first human grafts performed on limited skin areas are encouraging.

## ACKNOWLEDGEMENTS

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# The erythemal response to ultraviolet radiation in subjects with polymorphic light eruption

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## SUMMARY

A reflectance instrument was used to measure objectively the erythemal response to UV-B and UV-C radiation in subjects with polymorphic light eruption. The characteristics of the dose-response curves obtained for each quality of ultraviolet radiation were significantly different, but were essentially indistinguishable from the comparable dose-response curves measured previously in normal subjects. It is concluded that in this photodermatosis the chromophore(s) and mediator(s) of delayed (24 h) ultraviolet-induced erythema are identical to those in normal skin.

Polymorphic light eruption (PLE) is a relatively common idiopathic photodermatosis in which papular or vesicular lesions or both, develop in response to sun exposure, usually during the spring and early months of summer. Photobiological investigations of this disease have been concerned either with determination of the minimal eythema dose (MED) at different wavelengths using an irradiation monochromator (Magnus, 1964; Frain-Bell *et al.*, 1973), or with artificial provocation of the eruption using one or more exposures of fairly high doses (3–5 MEDs) of ultraviolet (UV) radiation from unfiltered mercury arc lamps or xenon lamps (Epstein, 1980; Jansen, 1982), or high doses of UV-A radiation (~100 J/cm$^2$) from filtered metal halide lamps (Hölzle *et al.*, 1982).

The minimal erythema dose at different wavelengths is often normal in subjects with PLE (Magnus, 1976) and, in our experience, monochromatic irradiation studies are not as helpful in establishing a diagnosis as they are in other photosensitive disorders, e.g. chronic actinic dermatitis (Diffey, Farr & Ive, 1984a). However, the MED represents only a single point, the detection threshold, on the UV-dose erythema-response curve. Reports of abnormal erythemal or morphological responses to high doses of UV radiation in PLE (Epstein, 1980; Jansen, 1982)

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suggest there may be a difference in the slope of the dose-response curve for UV-induced erythema in subjects with PLE, compared with normal individuals.

The development of a reflectance instrument to quantify erythema (Diffey, Oliver & Farr, 1984b) has allowed the characteristics of the dose response curves for 300 nm (UV-B) and 254 nm (UV-C) radiation to be determined in normal individuals (Farr & Diffey, 1985). This study clearly showed that the erythema elicited with UV-B radiation increased much more rapidly for a given fractional increment in radiant exposure than did UV-C induced erythema and it was concluded that the difference in the slopes of the response to these two wavelengths (254 and 300 nm) suggested that more than one mechanism is involved in the causation of ultraviolet erythema. We report now on the response to UV-B and UV-C radiation in subjects with PLE to compare the mechanisms of erythema production with those found in normal subjects.

## METHODS

### *Subjects*

Seven female Caucasian volunteers (mean age 36 years; range 26–52 years) with clinically diagnosed polymorphic light eruption were studied during January and February. Each subject had described several episodes of a papulo-erythematous rash accompanied by marked itching occurring principally on the chest and upper limbs, but sometimes on the face, hands and legs, following 1 or 2 h exposure to summer sunlight. The latent period between the end of exposure and the onset of the rash varied between a few hours and 2 days, and once present the rash would often last for a week or more.

### *Photo-irradiation apparatus and radiation dosimetry*

Three different optical radiation sources were used, the first for determination of minimal erythema doses (MED) and the latter two for dose-response studies.

(i) An irradiation monochromator (Diffey *et al.*, 1984a) was used for the determination of MEDs at 300 nm (full bandwidth at half maximum intensity of 5 nm) and 350 nm (bandwidth of 30 nm). Irradiance was measured using a thermopile incorporating a 2 mm square sensitive element.

(ii) A germicidal lamp (Philips type TUV 15W) was used for dose-response studies at 254 nm. Irradiance was measured with the thermopile.

(iii) A fluorescent sunlamp (Philips type TL20W/12) filtered by a cellulose triacetate plastic film (Bexford Ltd., type 51T35, 125 $\mu$m thick ) was also used. This results in a spectral power distribution from 290 to 400 nm, peaking at 310 nm and an erythemally-effective spectrum confined virtually to the UV-B wavelengths (290–320 nm). Radiation from this source is hereafter termed '300 nm radiation'. The irradiance from the optically filtered lamp was measured using a solar-blind vacuum phototube and multilayer interference filter combination calibrated at a wavelength of 300 nm and with a spectral response closely approximating to the erythema action spectrum for human skin.

The germicidal lamp and fluorescent sunlamp were each housed inside a luminaire which allowed simultaneous exposure of five skin sites in a graded series of doses (Diffey & Oliver, 1985). Wire strips or meshes incorporated into the luminaire acted as optical attenuators and allowed a graded series of doses to be achieved for a single irradiation time.

### *Minimal erythema dose*

A series of exposures to 300 and 350 nm radiation was administered to the skin of the upper back using the irradiation monochromator. The irradiation field was 20 mm$^2$ and a geometric progression of $\sqrt{2}$ was used for the dose increments. The exposure sites were examined at 24 h and the MED was taken as the lowest dose which produced minimally perceptible redness.

### *Dose-responses*

Adjacent circular sites (20 mm diameter) were marked on the back of each subject. Erythema was measured using a reflectance instrument (Diffey *et al.*, 1984b) which obtains an 'erythema index' related to the blood content of the subpapillary vascular plexus. Prior to irradiation, three measurements of the erythema index were made at each of the sites, with the subject lying prone on a couch. An approximate geometric series of increasing exposures was used: five exposures at 254 nm (germicidal lamp) were given with a dose increment of about 2; and ten exposures of 300 nm (fluorescent sunlamp) were given with a dose increment of about $\sqrt{2}$. The maximum exposure doses given to each subject were 2·8 and 1·2 kJ/m$^2$ at 254 and 300 nm, respectively. Twenty-four hours after irradiation, measurements of erythema index were repeated three times at each site. The increase in vasodilation due to the radiation was expressed as the difference between the mean post- and pre-irradiation erythema index at each site. This value has been shown to be a more reliable indicator of vasodilation than the post-irradiation erythema index alone (Farr & Diffey, 1984).

## RESULTS

### *Minimal erythema doses*

The MEDs recorded in each subject are given in Table 1. It can be seen from Table 2 that the median values for this group of subjects at both 300 and 350 nm are essentially the same as we reported previously for normal subjects (Diffey *et al.*, 1984a).

### *Dose-response*

The increase in erythema index was plotted against the logarithm of the UV radiation dose for each lamp and subject. An example of the response at 254 and 300 nm in a single subject is shown in Fig. 1. At doses in excess of the MED, an approximately straight line response was obtained. Linear regression was applied to the straight line part of the curve, selecting the limits of fitting by eye. By this process, a slope was calculated for each response, and analysis of variance applied

TABLE 1. The minimal erythema dose (MED) and slopes (mean ± 1 s.e.) of dose-response curves in each subject

| | MED (J/m$^2$) | | Slope of dose-response curve | |
|---|---|---|---|---|
| Subject | 300 nm | 350 nm | 254 nm | 300 nm |
| 1 | 560 | 2·8 × $10^5$ | 0·062 ± 0·008 | 0·253 ± 0·018 |
| 2 | 200 | 2·0 × $10^5$ | 0·065 ± 0·003 | 0·212 ± 0·008 |
| 3 | 140 | 2·0 × $10^5$ | 0·082 ± 0·010 | 0·226 ± 0·011 |
| 4 | 280 | 1·4 × $10^5$ | 0·058 ± 0·004 | 0·244 ± 0·015 |
| 5 | 560 | 4·0 × $10^5$ | 0·091 ± 0·007 | 0·177 ± 0·012 |
| 6 | 400 | 2·8 × $10^5$ | 0·081 ± 0·005 | 0·212 ± 0·013 |
| 7 | 280 | 2·8 × $10^5$ | 0·092 ± 0·004 | 0·177 ± 0·013 |
| Geometric mean | 310 | 2·4 × $10^5$ | 0·072 | 0·214 |

TABLE 2. Summary of median MEDs and pooled slopes of dose response curves in normal and PLE subjects

| Parameter | Normal subjects* | PLE subjects |
|---|---|---|
| MED (J/m$^2$) at 300 nm | | |
| Median | 280 | 310 |
| 95% Confidence limits | 140–660 | 110–870 |
| MED (J/m$^2$) at 350 nm | | |
| Median | $2{\cdot}7 \times 10^5$ | $2{\cdot}4 \times 10^5$ |
| 95% Confidence limits | $1{\cdot}4 \times 10^5$–$6{\cdot}4 \times 10^5$ | $1{\cdot}2 \times 10^5$–$4{\cdot}7 \times 10^5$ |
| Pooled slope of dose-response curve (mean ± s.d.) | | |
| 254 nm | 0·068 ± 0·001 | 0·072 ± 0·002 |
| 300 nm | 0·211 ± 0·006 | 0·214 ± 0·005 |

* Data taken from Diffey *et al.* (1984a); Farr & Diffey (1985); and P.M. Farr & B.L. Diffey (unpublished observations).

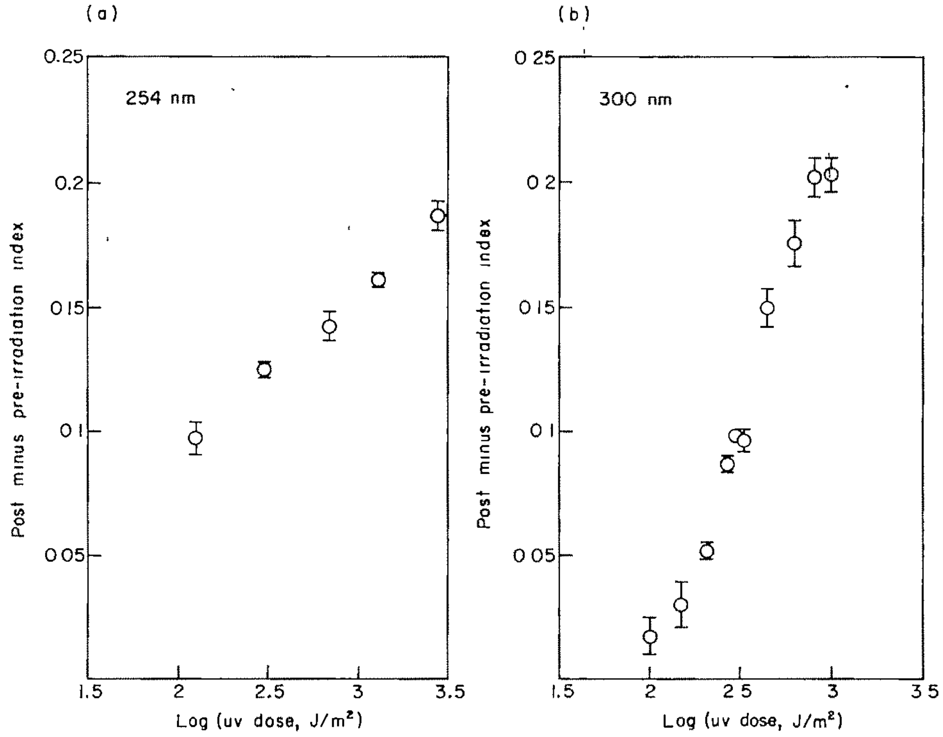

FIGURE 1. An example of the dose-response curves at (a) 254 nm and (b) 300 nm in a single subject. The error bars represent ± 1 s.d. of the measured difference between the post- and pre-irradiation erythema indices.

to regression (Armitage, 1971) was used to calculate the pooled slope for the group. The slopes of the linear part of the dose-response curves for each subject following exposure to 254 and 300 nm radiation are listed in Table 1. Non-parametric analysis (Wilcoxon's rank sum test) showed no significant difference between the slopes obtained at either 254 or 300 nm in this group of subjects and those measured previously in normal subjects (Farr & Diffey, 1985).

## DISCUSSION

The results of this study support the experience of others that the MED is usually normal or even increased in patients with PLE (Epstein, 1962; Magnus, 1964; Frain-Bell *et al.*, 1973). Although most of the subjects in our study exhibited palpable erythema with the higher doses of UV-B and UV-C when seen 24 h after irradiation, none of them showed multiple small papular lesions resembling the morphological changes that they described following exposure to sunlight. The only observed morphological change was a 'peau d'orange' appearance at the sites exposed to high doses of radiation of either wavelength. Although this phenomenon has been interpreted as an abnormal vesicular response (Jansen 1982), in our experience it is frequently observed in normal subjects whenever high enough doses of UV radiation are employed. In common with other workers (Tanenbaum *et al.*, 1981) we attribute this appearance to perifollicular oedema and regard it as a normal response. The difference in the slopes of the dose-response curves for UV-C and UV-B erythema in normal subjects suggests that more than one mechanism is involved in the causation of ultraviolet erythema (Farr & Diffey, 1985). The fact that the characteristics of the erythemal response to both UV-C and UV-B radiations in subjects with PLE do not differ appreciably from those found in normal subjects suggests that, in this disease, the chromophore(s) and mediator(s) of delayed (24 h) UV-induced erythema are identical to those in normal skin.

It is questionable whether the fairly high doses (3–5 MEDs) of broad-spectrum UV (Epstein, 1980; Jansen, 1982) or high doses of UV-A radiation (Hölzle *et al.*, 1982), which apparently result in morphological lesions characteristic of PLE, are climatologically realistic. Polymorphic light eruption typically occurs in the late spring or early summer each year following a few hours exposure to sunlight and very rarely is the eruption accompanied by severe sunburn. In view of this and the normal erythemal response in subjects with PLE, it is suggested that attempts to reproduce the rash as an aid to diagnosis should be made using light sources with a spectrum modified to simulate sunlight as closely as possible and repeated exposures given similar to those experienced naturally.

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# Immunological findings during treatment of multiple keratoacanthoma with etretinate

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## SUMMARY

Treatment with etretinate in a patient with multiple keratoacanthoma is described. Clinical improvement correlated with normalization of IL-2-production and mitogen induced lymphocyte proliferation. A causal relationship between reduced IL-2-production and eruption of keratoacanthoma is suggested.

Beurey *et al.* (1984) reported on the clinical course of a large keratoacanthoma in a child undergoing treatment with etretinate. Sterry *et al.* (1981) reported on the effective treatment of eruptive keratoacanthoma in a 74-year-old woman, with the aromatic retinoid Ro 10-9359. Haydey *et al.* (1980) discussed a case of keratoacanthoma in a 43-year-old male, that was successfully treated by surgery and subsequent oral administration of 13-*cis*-retinoic acid. Braun-Falco *et al.* (1982) observed a prophylactic effect of etretinate in xeroderma pigmentosum.

We have used etretinate to treat a case of multiple keratoacanthoma in a 69-year-old female patient. The results of etretinate treatment on the keratoacanthomas, and immunological findings in the course of treatment are reported.

## CASE REPORT

A 69-year-old female patient with a 2-year history of multiple, partly painful skin tumours of 3 mm to 3 cm in diameter was admitted to our hospital in February 1983. The tumours were hyperkeratotic, partly erosive and distributed over face, arms (Fig. 1), trunk and legs. A painful ulcerated tumour 3 cm in diameter was located on the dorsum of the left hand (Fig. 2). Clinical and histological findings substantiated the diagnosis of multiple keratoacanthoma. A carcinoma of the rectum was diagnosed in 1978. A colostomy was performed, which the patient has managed without difficulty. Keratoacanthoma developed in 1981 and showed no signs of

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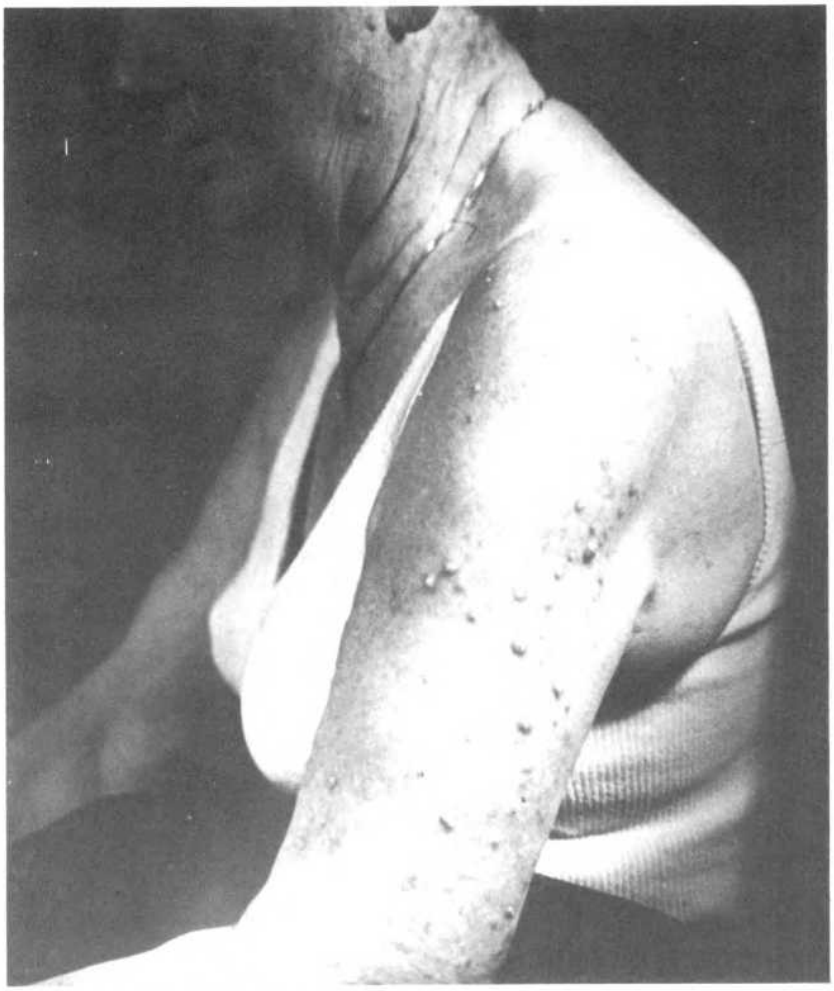

FIGURE 1. Before treatment: multiple keratoacanthoma on the upper arm.

spontaneous regression. The tumours increased in number and size continuously despite topical treatment with tretinoin over a 2-year period.

Treatment was initiated with 40 mg etretinate per day. A substantial subjective improvement, the cessation of pain and drying up of the erosions, occurred after one month of treatment.

Initial tumour regression was observed after 2 months (Fig. 3). The large tumour on the dorsum of the left hand disappeared completely after 6 months of treatment (Fig. 4). The patient suffered from the various known side-effects of etretinate treatment (including dry lips and hair loss). In addition, oedematous swelling of the cheeks led to ectropion of both lower eyelids. The side effects necesitated discontinuation of therapy after 7 months. New tumours developed after a period of 3 weeks. Therefore, treatment was recommenced at 20 mg/day. This dose was not sufficient to suppress further keratoacanthoma formation. Etretinate treatment was continued at 40 mg/day for another 14 months. This dosage was sufficient to suppress general tumour formation, although a few tumours arose sporadically.

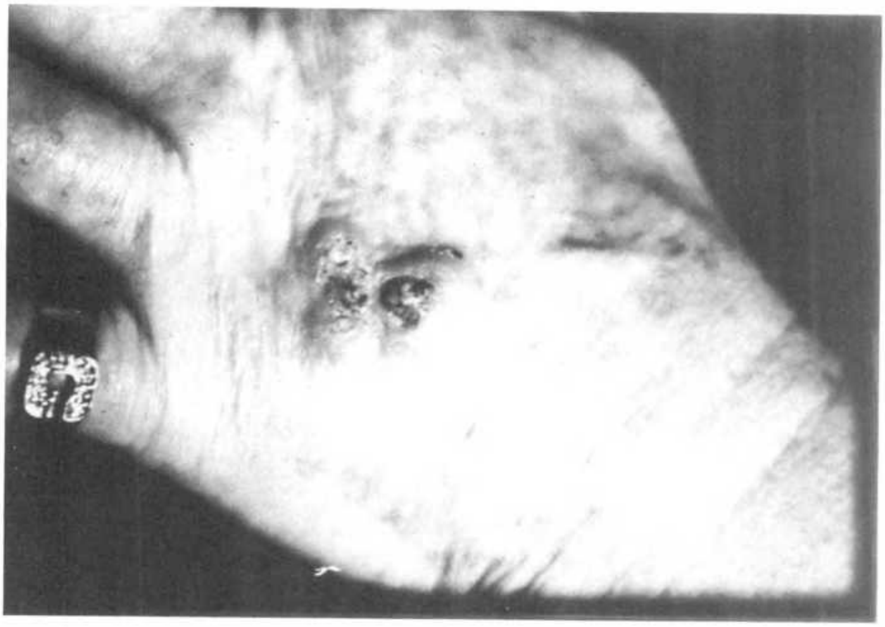

FIGURE 2. Before treatment: keratoacanthoma (3 cm in diameter) with erosions and local inflammation on the dorsum of the left hand.

## METHODS

The following immunological parameters were examined before treatment, during therapy and after clinical remission.

(1) Immunofluorescent examination of peripheral lymphocytes by means of monoclonal antibodies (total T cells, T-helper, T-suppressor, monocytes, B cells).

(2) Mitogen induced lymphocyte stimulation (two T-cell mitogens: PHA and ConA).

(3) Interleukin-2 production.

### *Lymphocyte isolation*

Peripheral blood mononuclear cells from the patient and from healthy volunteers were isolated by Ficoll-Isopaque density gradient sedimentation according to the method of Bøyum (1968).

### *Lymphocyte subsets*

Analysis of cell markers with monoclonal antibodies was performed by an indirect immunofluorescence assay according to the method of Reinherz *et al.* (1979). The monoclonal antibodies (Ortho-Diagnostic System, Raritan, N.Y.) used were: OKT3 that reacts with mature human T-lymphocytes, OKT4 that reacts with the inducer/helper subpopulation, OKT8 that reacts with the cytotoxic/suppressor subpopulation, OKM5 that reacts with monocytes and OKB7 that reacts with B-cells. Lymphocytes, $5 \times 10^5$, were incubated with 10 $\mu$l monoclonal antibody (diluted 1 : 5) at 4° C for 30 min and subsequently washed three times. They were resuspended in FITC-labelled goat-antimouse $(Fab')_2$ Ig (Tago Inc., Burlingame CA 94010, U.S.A.) at 4° C for 30 min and then washed thoroughly. Smears were examined with a Zeiss fluorescence microscope with epi-illumination. Two hundred cells were counted by phase contrast.

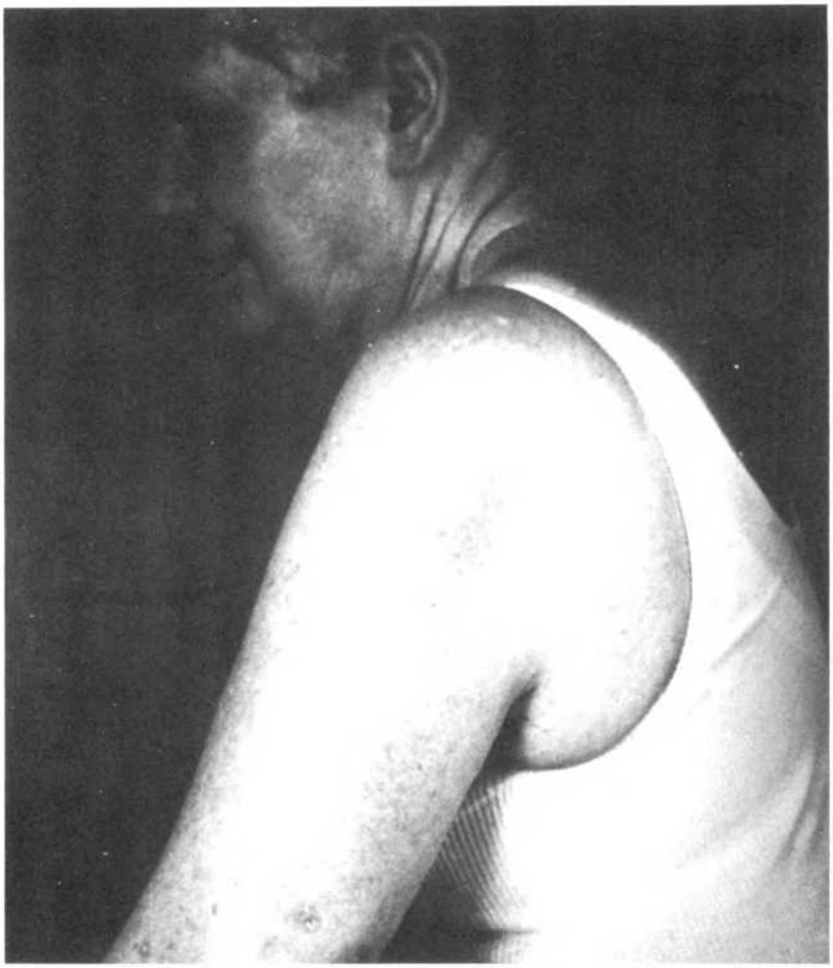

FIGURE 3. After 3 months of treatment with 40 mg etretinate/day.

Numbers of fluorescence-positive cells were recorded, and the different subpopulations were expressed as absolute values and as percentages.

The negative control was composed of cells that were incubated with the second antibody in the absence of monoclonal antibody, after treatment with normal mouse serum.

*Mitogen induced lymphocyte proliferation*

An assay for mitogen induced lymphocyte proliferation was performed as previously described (Diamantstein, Blitstein-Willinger & Schulz, 1974). Briefly, 0·15 ml Ficoll–Isopaque separated cells ($2 \times 10^5$/ml) were suspended in a complete medium (CM) composed of RPMI 1640 medium, supplemented with 5% heat inactivated (56°C for 30 min) mitogen and mycoplasma pre-tested FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Fa. Seromed, Munich, F.R.G.).

Samples were incubated in triplicate in Polystyrol microtitre plates (Greiner, Nürtlingen,

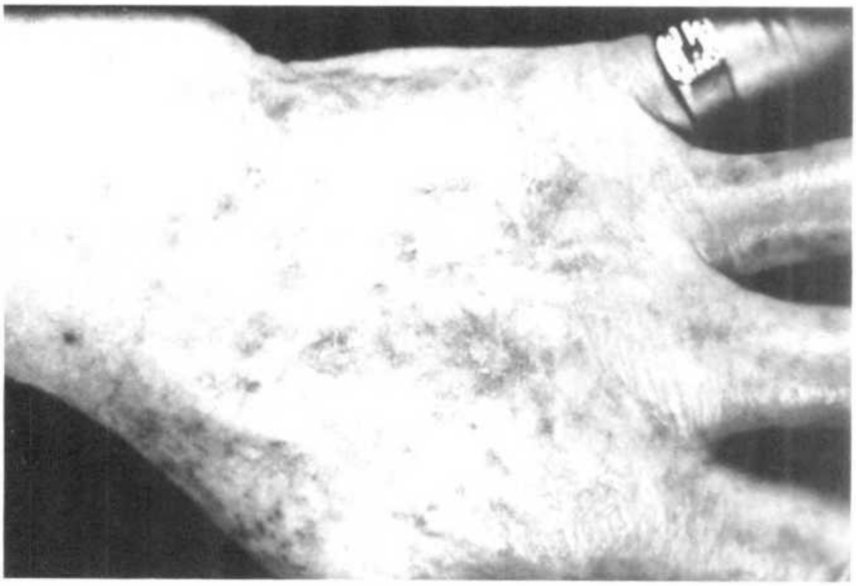

FIGURE 4. Complete tumour regression (compare with Fig. 2) after 6 months.

F.R.G.) with (a) CM; (b) different concentrations of PHA (Seromed, Munich, F.R.G.); and (c) different concentrations of ConA (Seromed, Munich, F.R.G.). The cells were cultured for 72 h at 30°C in 5% CO2 and were pulsed for the last 4 h with 0·2 $\mu$Ci/ml $^3$H-thymidine (5 Ci/mmol, Amersham International, U.K.). The cells were then harvested on to glass filter strips with the aid of a multiple automated sample harvester. Incorporated thymidine was counted on a Beckman liquid scintillation counter. Cell proliferation was expressed in cpm ± S.D. of triplicate cultures.

### *Assay for IL-2 activity*

Ficoll-Isopaque purified cells $1 \times 10^6$/ml were cultivated with and without 10 $\mu$l PHA/ml in CM supplemented with 2% FCS. The supernatants were harvested after 48 h and passed through a 0·22 $\mu$m millipore filter (Millex-Or, Millipore Sa Molsheim, France). PHA was removed from supernatants by absorption with chicken red blood cells according to the method of Bonnard, Yasaka and Jacobsen (1979).

The supernatants were tested for IL-2 activity as described by Gillis, *et al.* (1978). However, we used $1 \times 10^6$/ml lymphocytes from healthy young volunteers as responder cells. The cells were pre-incubated with 5 $\mu$g PHA/ml in CM supplemented with 10% FCS for 7 days. The responder T-cells were washed three times and resuspended at a density of $4 \times 10^5$/ml viable blasts in CM with 5% FCS. Viability was determined by trypan blue exclusion. $3 \times 10^4$ cells/75 $\mu$l were cultivated with 75 $\mu$l/well of CM, with different doses of PHA and/or with a $\log_2$ dilution series of the supernatants. Controls were incubated with Lymphocult T charge 113053 (Biotest Institute GmbH, Frankfurt, F.R.G.) as a standard for IL-2. Cells were cultivated for 3 days and $^3$H-Tdr uptake was measured as described above. Cells from at least one healthy subject were tested alongside those from the patient.

## RESULTS

Cells cultured in the absence of IL-2 were 95% trypan blue positive and only incorporated control levels of $^3$H-thymidine. IL-2 activity was quantified by probit analysis according to Gillis *et al.* (1978). Results were expressed in units/ml (U/ml). PHA did not stimulate IL-2 activity in responder T cells.

Before treatment, lymphocyte subpopulations in the patient were not significantly different from the healthy control group (Table 1). However, lymphocyte proliferation following mitogen stimulation was substantially reduced. The PHA response was 25% of that in an age matched control and the ConA response 40% (Fig. 5).

TABLE 1. Distribution of T-cell subpopulations before and after treatment

| Lymphocytes | Controls* | Patient before therapy | Patient after therapy |
|---|---|---|---|
| Total | 98% (1570 ± 363) | 97% (1800) | 98% (2500) |
| OKT3 + T-cells | 60–90% (1260 ± 273) | 82% (1440) | 80% (2000) |
| OKT4 + $T_H$ cells | 40–60% (800 ± 118) | 54% (900) | 50% (1250) |
| OKT8 + $T_S$ cells | 15–30% (420 ± 133) | 28% (504) | 30% (750) |
| OKM5 + cells | 10–25% (350 ± 58) | 15% (270) | 10% (250) |
| OKB7 + cells | 5–15% (130 ± 38) | 10% (180) | 15% (375) |

* Mean of 10 healthy subjects.
Values in parentheses are absolute counts.

This reduced rate of lymphocyte proliferation could not be accounted for by a diminished number of T cells or their sub-populations (Table 1). However, whilst the relation of the different T cell sub-populations to one another did not change, the total lymphocyte count increased after remission. The T cell growth factor (IL-2) is in part responsible for antigen–lectin induced clonal expansion of T cells; therefore IL-2 production was measured. The patient had substantially reduced IL-2 production before treatment (Fig 5). In order to determine whether mitogen induced lymphocyte transformation and IL-2 production were affected by etretinate treatment, thymidine uptake and IL-2 production were measured after clinical remission (Fig. 6). There were no changes in the unstimulated levels after treatment. Cells cultivated with culture medium only, incorporated control levels of $^3$H-thymidine. Spontneous IL-2 production was not observed before or after treatment.

## DISCUSSION

Solitary keratoacanthoma (KA) are frequent; multiple keratoacanthoma are rare. Joint occurrence of multiple KA and internal malignant neoplasms has been reported (Snider & Benjamin, 1981; Kingman & Callen, 1984). Degos *et al.* (1967) and Weber *et al.* (1970) reported the relatively rapid development of keratoacanthoma in patients with leukaemia. Furthermore, Walder *et al.* (1971) noticed that several patients undergoing immunosuppressive therapy developed multiple KA. Claudy & Thivolet (1975) noticed the same phenomenon and

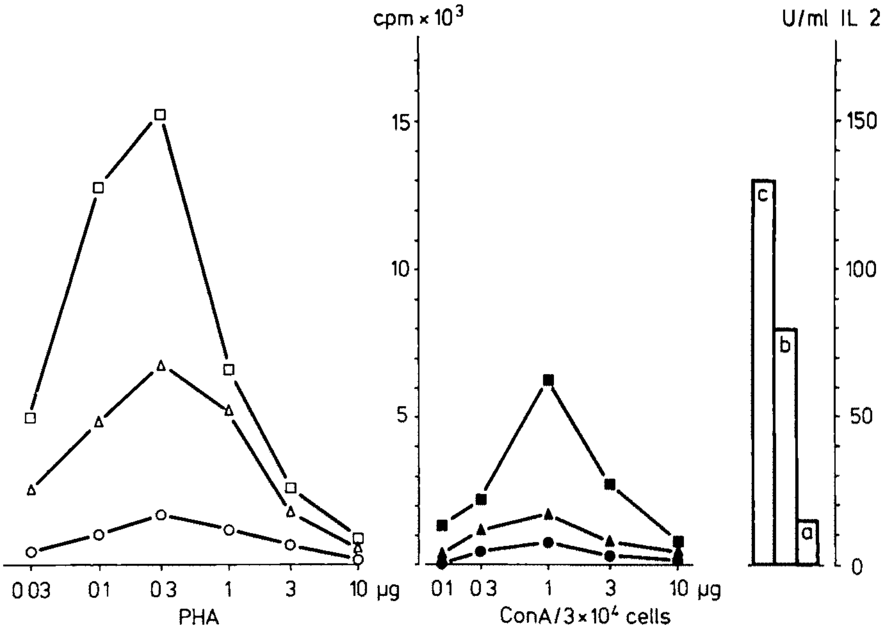

FIGURE 5. Immune status before treatment. Mitogen induced lymphocyte proliferation and IL-2 production.

| PHA | ConA | |
|---|---|---|
| ○ | ● | 69-year-old patient (a) |
| △ | ▲ | 70-year-old healthy control (b) |
| □ | ■ | 40-year-old healthy control (c) |

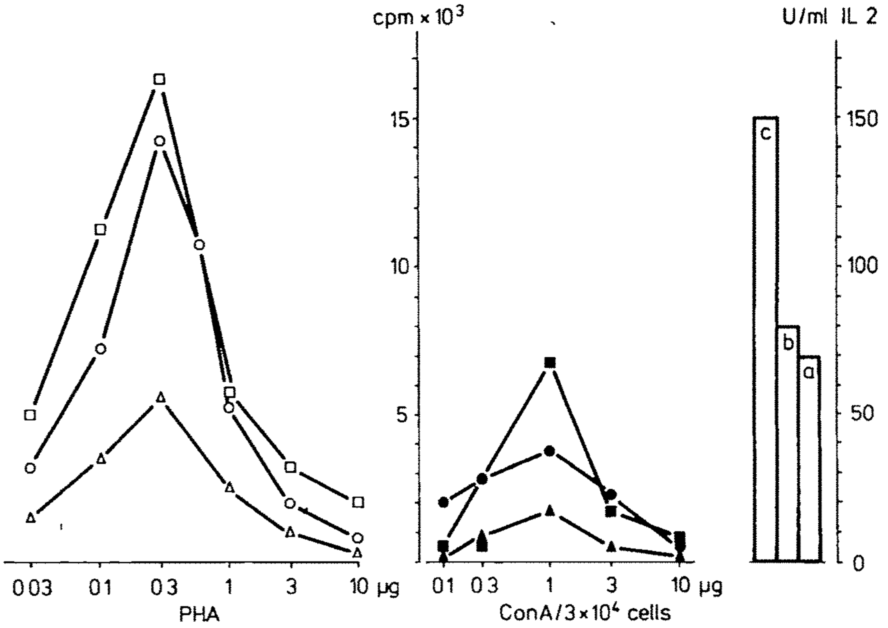

FIGURE 6. Immune status after 11-months treatment: mitogen induced lymphocyte proliferation and IL-2 production.

| PHa | ConA | |
|---|---|---|
| ○ | ● | 70-year-old patient (a) |
| △ | ▲ | 70-year-old healthy control (b) |
| □ | ■ | 25-year-old healthy control (c) |

suggested that immunosuppressives should be avoided in patients with keratoacanthoma for fear of aggravating the condition. The treatment of multiple KA by cryotherapy or by topical application of cytostatic agents has remained unsatisfactory (Snider & Benjamin, 1981). Reports of successful treatment of keratoacanthoma with etretinate prompted us to initiate this form of therapy. Although a regression of tumour growth was observed, the sporadic development of new tumours could not be halted. It is conceivable that a new internal malignancy or metastases were responsible for maintaining this condition, although screening procedures gave no evidence of malignant growth.

The possible effects of therapy on immunological parameters was examined during the course of therapy, IL-2 production and mitogen induced lymphocyte proliferation both increased considerably during etretinate treatment. This augmentation corresponded with the improvements in the clinical condition. During the attempt to reduce etretinate medication to 20 mg/day because of severe side-effects, we not only observed a distinct increase in KA development after 4 weeks, but also a substantial decrease in IL-2 production and mitogen induced lymphocyte proliferation (data not presented). Whether a causal relationship exists between IL-2 deficiency and the formation of keratoacanthoma, remains unanswered. Further *in vitro* and clinical studies are necessary.

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# Egg and milk exclusion diets in atopic eczema

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## SUMMARY

Fifty-three patients with atopic eczema took part in a double blind controlled cross-over trial of an egg and cow's milk exclusion diet. Response to the diet was assessed in terms of areas affected, day and night time itch, and topical steroid usage.

Twenty-five percent of the patients failed to comply adequately with the trial regime and were excluded from the analysis. Of the remaining 40 patients, ten appeared to benefit from the diet and were advised to continue egg and milk avoidance. This response rate to the diet was not statistically significant.

Atopic eczema is a common and disabling condition in childhood which may persist into adult life. The cause is not known though numerous aggravating factors and possible aetiological agents have been proposed. Recently, interest has again centred on the aetiological role of dietary factors (Hammer, 1977; Juto, Engberg & Winberg, 1978), but there have been few adequately controlled trials until Atherton *et al.* (1978) carried out a double blind cross-over trial and demonstrated that as many as 60% of an unselected group of children with atopic eczema benefited from a diet which excluded egg and milk. Although this work is well known it is still not widely accepted by clinicians and, perhaps as a result of this, patients are often left to experiment with unsupervised diets at home.

The aim of this study was to confirm this work, with certain modifications, in children, and also to assess the effect of the diet in older patients.

## METHODS

### *Patients*

The 53 patients (29 female and 24 male) were drawn from regular attenders at our outpatients department. All had active atopic eczema which required regular conventional treatment with emollients, topical steroids and oral antihistamines. They were drawn from the local cosmopolitan population, 25 patients (47%) being non-Caucasian in origin. On admission into

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the trial the patients were divided into three age groups, age 1–7 years (23 patients), age 8–15 (12 patients and 16–23 years (18 patients). Informed consent was obtained from all patients and/or parents before the start of the trial.

The trial was divided into three 6-week periods (Fig. 1). During the first and third periods patients taking an egg and milk exclusion diet received either a soya-based milk substitute (trial period) or an egg and cow's milk preparation (control period). During the middle period patients resumed a normal diet, including egg and milk if these foods were usually avoided for any reason.The order in which the 'milk substitutes' were taken was randomly allocated, patients were asked to drink a pint a day and were unaware of the different nature of these substances. Both milks were similar in appearance and equally unpleasant to taste, the egg and cow's milk preparation contained the equivalent of one egg/pint of milk.

FIGURE 1 Plan of trial.
Patients were randomly allocated to either (1) or (2)

| | | | |
|---|---|---|---|
| (1) | Exclusion diet + soya milk (trial period) | Normal diet | Exclusion diet + egg & cows milk (control period) |
| (2) | Exclusion diet + egg & cows milk (control period) | Normal diet | Exclusion diet + soya milk (trial period) |
| | ◄—— 6 weeks ——► | ◄— 6 weeks —► | ◄—— 6 weeks ——► |

A full dietary history was obtained from each patient who was then instructed by the dietician (J.A.B.) in a diet which excluded all foods containing egg and cow's milk. Chicken and beef were also excluded as they may contain proteins common to egg and milk.

Throughout the trial patients were asked to continue their usual treatment and on a diary/score card (Fig. 2) were asked to note day and night time itch and sleep disturbance using a 10-cm scale. Intake of oral antihistamine (usually trimeprazine at night) was also recorded plus any dietary lapses and an estimate of their consumption of milk substitute each day. Weighed tubes of the patients' normal topical steroid were provided on each visit and were to be returned at the end of each trial period for further weighing. Topical steroids used varied between individuals, most used 1% hydrocortisone cream (± 10% urea), clobetasone butyrate 0·05% or dilutions of beclomethasone dipropionate 0·025%.

At the beginning of the trial and at the end of each 6-week period the extent and activity of the eczema was graded independently by two observers (V.S.N. and R.A.M.). The body surface was divided into 20 zones using the method of Atherton *et al.* (1978), and the presence of acute eczema (erythema and vesiculation) and chronic eczema (lichenification and prurigo papules) was noted, both on a scale from 0 to 3. The area score (sum of the two observers) was graded to assess severity on admission to the trial 0–30 mild, 30–60 moderate and 60–90 severe.

At the start of the trial patients were prick tested to house dust mite, grass pollen, cat fur, whole egg and whole cow's milk (Bencard), and blood was obtained from 46 patients for total IgE estimation and RAST tests to a similar range of antigens (Pharmacia).

FIGURE 2. Patient diary score card.

PATIENT DIARY CARD

NAME ... . .. ....

DATES COVERED.
From.. . . . .
To . . ......

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Date | | | | | | | | | | | | | | | | | | | | | |
| Day-time itch (0–10) | | | | | | | | | | | | | | | | | | | | | |
| Night-time itch/ Sleep disturbance (0–10) | | | | | | | | | | | | | | | | | | | | | |
| Antihistamines<br>Tablets/day<br>Name. ......... | | | | | | | | | | | | | | | | | | | | | |
| Dietary lapses<br>What food and how much? | | | | | | | | | | | | | | | | | | | | | |
| Amount of milk substitute taken | | | | | | | | | | | | | | | | | | | | | |
| New tube of ointment?<br>Yes or No | | | | | | | | | | | | | | | | | | | | | |

*Recording of Symptoms*

Day-time Itch

0 1 2 3 4 5 6 7 8 9 10

Please grade from:
0 = No itch at all
10 = Unbearable itch

Sleep Disturbance

0 1 2 3 4 5 6 7 8 9 10

Please grade from
0 = Best night ever
10 = Unable to sleep

Thank you for your help

TABLE 1. Clinical data

| | | Total group (53 patients) | Non-compliers (13 patients)* | Responders (10 patients)* | Non-responders (30 patients)* |
|---|---|---|---|---|---|
| Age (years) | 1–7 | 23 | 9 (39%) | 5 (22%) | 9 (39%) |
| | 8–15 | 12 | 1 (8%) | 3 (25%) | 8 (66%) |
| | 16–32 | 18 | 3 (17%) | 2 (11%) | 13 (72%) |
| Sex | Female | 29 | 9 | 5 | 14 |
| | Male | 24 | 4 | 5 | 16 |
| Race | Non-Caucasian | 25 | 10 | 2 | 13 |
| Severity on admission to trial | Mild | 11 | 2 (18%) | 1 (9%) | 8 (73%) |
| | Moderate | 23 | 7 (30%) | 4 (17%) | 12 (53%) |
| | Severe | 19 | 4 (21%) | 5 (26%) | 10 (53%) |

* Figures quoted in parentheses refer to percentages of the total group.

## RESULTS

Twelve patients were excluded from the analysis because of their failure to comply adequately with the trial, usually because they found the diet was too difficult to adhere to. Seven of these patients withdrew voluntarily, five during or following the 'trial diet' and two following the 'control diet', none of them apparently having benefited from the diet. Five patients were excluded for dietary lapses. Of these, three may have been helped by the diet, but could not be assessed because of poor compliance during the trial.

One patient who appeared unable to tolerate the egg/milk powder, developed gastro-intestinal symptoms followed by itch and exacerbation of eczema. She did not complete the trial, but was advised to continue with an egg- and milk-free diet. Of this total of 13 patients excluded from analysis, nine were in the youngest age group and ten were from the non-Caucasian families (Table 1).

Forty patients completed the trial, and from these patients the following data were compared for each trial period:

(i) area score (the sum of the two observers);
(ii) total itch score (the sum of day and night time itch, excluding the 1st week of each trial period from the analysis);
(iii) topical steroid consumption (grams of fluorinated steroid and grams of 1% hydrocortisone).

As antihistamines were not regularly used by the majority of patients, their consumption was excluded from the analysis.

Ninety-five per cent confidence intervals using the *t* distribution were calculated for the differences. No statistically significant difference was shown between the scores at the end of the 'trial diet' compared with those at the end of the normal diet (Table 2) or between the scores at

TABLE 2. Data analysis for all patients who completed the trial

| | Trial diet | | | Normal diet | | |
|---|---|---|---|---|---|---|
| | Mean | Min | Max | Mean | Min | Max |
| Area score | 48 | 6 | 116 | 49 | 12 | 120 |
| Itch score | 250 | 20 | 546 | 234 | 38 | 452 |
| Fluorinated steroid used (g) | 43 | 0 | 100 | 37 | 0 | 89 |
| Hydrocortisone used (g) | 27 | 0 | 242 | 21 | 0 | 195 |

| | Difference, trial diet – normal diet (mean) | 95% confidence interval |
|---|---|---|
| Area score | −1 | −6 to 3·4 |
| Itch score | 15 | −21 to 51 |
| Fluorinated steroid | 5 8 | 1 to 10 |
| Hydrocortisone | 6·0 | 0 1 to 12 |

TABLE 3. Data analysis for the responders only

| | Trial diet | | | Normal diet | | |
|---|---|---|---|---|---|---|
| | Mean | Min | Max | Mean | Min | Max |
| Area score | 33 | 6 | 58 | 51 | 20 | 77 |
| Itch score | 176 | 20 | 297 | 224 | 64 | 425 |
| Fluorinated steroid | 40 | 0 | 100 | 34 | 0 | 04 |
| Hydrocortisone | 20 | 0 | 100 | 13 | 0 | 05 |

the end of the trial diet compared with those at the end of the control diet. All the confidence intervals contained zero, but if anything patients did rather worse on the 'trial' diet than on their normal diet. Patients used rather more topical steroids while on the 'trial' diet. Any overall benefit from the 'trial' diet must be very small.

At the end of the study 10 of the 40 patients appeared to have improved on the trial diet and had lower area scores and itch scores than on the normal diet (Table 3) or on the control diet. Of those who responded, five were in the youngest age group and two were non-Caucasians. All ten responders were advised to carry on with egg and milk avoidance and six have done so with continued improvement in their eczema. Five of the ten who apparently responded to the diet were graded as having severe eczema on admission to the study and all had high IgE levels (Tables 1 & 4). No other aspect of the history or examination findings distinguished them from the non-responders. More responders had the trial diet first than the group as a whole.

Analysis of the prick test observations with the RAST test results and IgE levels (where obtained) was not generally helpful in predicting response to the trial. We confirmed the observation that positive prick tests to dietary antigens (egg and milk) are more often present in the younger patients (Van Asperen, Kemp & Mellis, 1985) as 43% of the 1–7 year-old group had positive prick tests to egg and/or milk (Table 4), but become less common with age as the inhalant allergens come to predominate. Ninety per cent of our patients over the age of 8 had positive prick tests to two or more inhalant allergens. Dietary history was not helpful in

TABLE 4. Results of investigations

| | Age (years) | Total group | Non-compliers | Responders | Non-responders |
|---|---|---|---|---|---|
| Positive prick tests to egg and/or milk | 1–7 | 10/23 (43%) | 4/9 (44%) | 3/5 (60%) | 3/9 (33%) |
| | 8–15 | 2/12 (17%) | 1/1 (100%) | 1/3 (30%) | 0/8 |
| | 16–32 | 2/18 (11%) | 1/3 (30%) | 0/2 | 1/13 (8%) |
| Positive prick tests to two or more inhalant allergens | 1–7 | 14/23 (61%) | 4/9 (44%) | 5/5 (100%) | 5/9 (55%) |
| | 8–15 | 11/12 (92%) | 1/1 (100%) | 3/3 (100%) | 7/8 (88%) |
| | 16–32 | 17/18 (94%) | 3/3 (100%) | 2/2 (100%) | 12/13 (92%) |
| IgE Level > 100 | | 26/46 (59%) | 5/11 (45%) | 7/7 (100%) | 14/28 (50%) |

predicting response to diet as only a trace of egg was included in the normal pre-trial diet of half of the patients in each group, whether responsive to the diet or not.

## DISCUSSION

Ten of the 40 patients who completed the trial may have benefited from egg and milk exclusion from their diet. (One additional child was intolerant of the egg and milk and was thus advised to continue with egg and milk avoidance.) This is a response rate of 25% and means we have failed to confirm any striking beneficial effect from egg and milk exclusion diets in unselected children and young adults with atopic eczema. The best response observed to the diet was in young Caucasians with severe eczema. Older patients, especially with mild eczema, appeared less likely to benefit.

These results suggest that the importance of dietary aspects of atopic eczema has been over emphasized in the past. An alternative explanation is that an egg and milk exclusion diet *per se* is too limited and that other more relevant food antigens were not excluded. Even soya, used by us as a non-antigenic alternative to cow's milk, is now known to produce allergic reactions (Taitz, 1982).

The prescription of an egg and milk exclusion diet on a routine basis in unselected patients with atopic dermatitis is likely to benefit only a few. Properly controlled studies of more rigorous exclusion diets tailored to the patient's needs are urgently required, with open, followed by double blind, challenges of foodstuffs in order to try to define the true role of dietry antigens in the pathogenesis of atopic eczema.

## ACKNOWLEDGMENTS

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## *Case Report*

# Pyoderma gangrenosum, subcorneal pustular dermatosis and IgA paraproteinaemia

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## SUMMARY

A patient with pyoderma gangrenosum, subcorneal pustular dermatosis and IgA paraproteinaemia is described.

The association of either pyoderma gangrenosum or subcorneal pustular dermatosis with IgA paraproteinaemia is well recognized. However, all these diseases occurring in the same individual is extremely rare, with only one reported case (Wolff, 1971). We describe a second case and review other rashes reported with pyoderma gangrenosum.

## CASE REPORT

A 52-year-old man had a two-month history of an itchy rash on his trunk and limbs which had progressively worsened two weeks prior to presentation. During this time, he had developed a tender nodule on his left ankle which rapidly enlarged and ulcerated. He felt well and had no other symptoms. He had been treated with erythromycin and amoxycillin for seven days without improvement and had taken no other drugs.

On examination there was a widespread symmetrical eruption consisting of groups of erosions and flaccid pustules up to 5 mm in diameter (Fig. 1). The skin around the base of each pustule was not inflamed. The rash was most prominent on the forearms, lower abdomen, groins and legs. The face and mucous membranes were not involved. Over the left lateral malleolus was a necrotic ulcer 5 cm in diameter with a purple, ragged undermined edge. There were no other abnormal findings.

Haemoglobin was 14·0 g/l WCC 11 2 × $10^9$/l and ESR 33 mm/h. Levels of bilirubin, transaminases, alkaline phosphatase, calcium, urea, creatinine, electrolytes, prothrombin time and activated partial thromboplastin time were normal. Anti-nuclear factor was weakly positive at a titre of 1:25 but anti-DNA antibodies were absent and complement levels were normal. Concentrations of immunoglobulins were IgG 10 g/l (normal 5·0–16·0 g/l), IgM 0·8 g/l (normal 0·5–1·7 g/l) and IgA 5 g/l (normal 1·2–4·2 g/l). Immunoelectrophoresis revealed a monoclonal

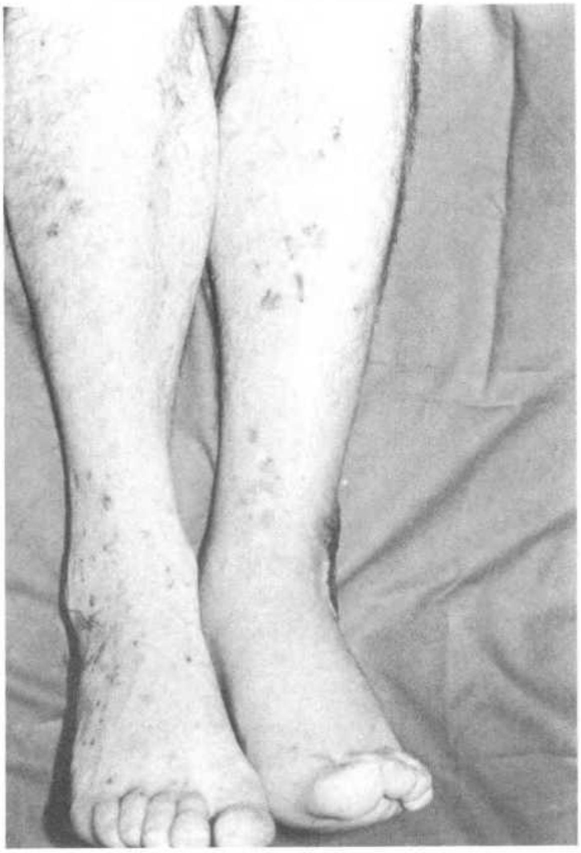

FIGURE 1. View of rash on legs.

IgA-kappa 3·0 g/l but no free light chains in the urine. There were no cryoproteins, and plasma viscosity was normal. Bone marrow aspiration showed an increased number of plasma cells (6% of total) and immunofluorescence of the marrow revealed increased immunoglobulin-secreting cells (15% of total), with the majority (95%) being IgA-kappa. A radiological skeletal survey was normal. Measurements of neutrophil opsonization of *Saccharomyces cerevisiae* and phagocytosis and killing of *Candida albicans* were normal. Cultures from pustules were negative.

Histology of the rash showed an infiltrate of neutrophils and eosinophils in the superficial dermis centred around blood vessels with swollen endothelial cells, and leukocytoclasis. Neutrophils infiltrated through into the epidermis where they collected in sub-corneal pustules (Fig. 2). Immunofluoresence showed only a sparse granular deposition of C3 throughout the dermo-epidermal junction. A biopsy from the advancing edge of the ulcer showed a dense infiltrate of neutrophils and eosinophils in the deep dermis with thrombosis of larger vessels and fibrinoid necrosis. The superficial vessels were dilated with swollen endothelial cells and were surrounded by an infiltrate of neutrophils. The epidermis was spongiotic, and immunofluorescence showed fine granular C3 in the superficial vessels and at the dermo-epidermal junction. Histology of clinically uninvolved skin was normal and there was sparse granular C3 at the dermo-epidermal junction.

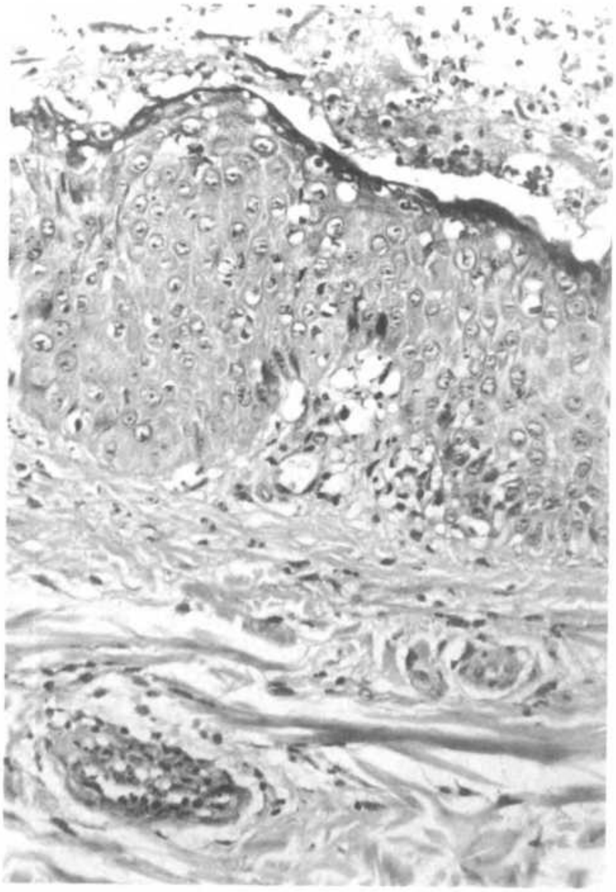

FIGURE 2. Neutrophils marginating along swollen endothelial cells and migrating through epidermis into a subcorneal pustule. (Original × 315.)

### *Treatment*

The patient was treated initially with dapsone 200 mg daily. Within 72 h there were no pustules and the rash had greatly improved. However, during this time the pyoderma gangrenosum enlarged from 37 $cm^2$ to 51 $cm^2$ and similar ulcers had developed in subcorneal pustules and biopsy sites. Therefore prednisolone 40 mg daily was added and increased to 80 mg daily two days later. The ulcers healed with scarring after three weeks' treatment; there was no scarring from the rash. During the subsequent five months, prednisolone and dapsone were discontinued without recurrence of the disease.

## DISCUSSION

Pyoderma gangrenosum and subcorneal pustular dermatosis are each associated with IgA paraproteinaemia with or without myeloma (Sneddon & Wilkinson, 1979; Powell *et al.*, 1983). However, there is only one previous report of all three diseases occurring together (Wolff, 1971) and it is unclear whether this association is coincidental.

Histology showed an acute neutrophilic vasculitis in the lower dermis at the advancing edge of the pyoderma, and similar but less severe changes directly beneath each subcorneal pustule. The adjacent dermis and epidermis were normal. Similarly to Wolff (1971) we found no IgA in the skin lesions. These features suggest that a vasculitis might be the cause of both ulcer and rash. There is evidence for this in pyoderma gangrenosum (Schroeter & Su, 1980; Powell *et al.*, 1983) and vasculitis may be a feature of subcorneal pustular dermatosis (Montgomery, 1967). In both diseases there is an infiltrate of polymorphs in the epidermis. There is insufficient histological information for comparison with the case of Wolff (1971); however, Andersen (1981) described pyoderma gangrenosum with a rash consisting of small crusted lesions and bullae. Histologically there were subcorneal pustules and a perivascular infiltrate of neutrophils in the underlying dermis. This patient also had an IgA paraproteinaemia and although the rash was described as 'pemphigus erythematosus-like', it seems very similar to our own case. In all these cases the rash responded to sulphones.

Other rashes have been reported with pyoderma gangrenosum. These include a blistering eruption described as atypical dermatitis herpetiformis because of its appearance and response to sulphapyridine (Perry & Brunsting, 1957), but without histological evidence of level of blister formation. One of these patients had rheumatoid arthritis but there was no associated disorder in the other two. A similar rash in two patients with pyoderma gangrenosum and rheumatoid arthritis was described by Ayres & Ayres (1958); histology of the lesions showed subcorneal and subepidermal vesicles containing polymorphs and an acute dense polymorph infiltrate in the underlying dermis. Other descriptions include a patient with pyoderma gangrenosum, IgA paraproteinaemia and atypical dermatitis herpetiformis (Powell *et al.*, 1983) and bullous lesions in a patient with IgG myeloma, which were vasculitic and developed into pyoderma gangrenosum (Horton, Trounce & MacDonald, 1984). In myeloid leukaemia there are reports of superficial pustules and bullae in association with pyoderma gangrenosum (Sheps, Schapero & Ramsay, 1978) of neutrophils invading the epidermis with subcorneal bullae at the advancing edge of the ulcer (Perry & Winkelman, 1972) and intra-epidermal bullae which developed into pyoderma (Burton, 1976).

There is insufficient evidence to conclude that these bullous and pustular eruptions associated with pyoderma gangrenosum are different expressions of the same inflammatory process. However, the histological similarities and the response to sulphones suggest this. The alternative explanation is that IgA paraproteinaemia, rheumatoid arthritis and leukaemia are each associated with a distinct bullous eruption and pyoderma gangrenosum. Further study to establish the range of lesions present in an individual, their evolution and cytological characteristics is necessary.

## ACKNOWLEDGMENTS

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### Neutrophilic spongiosis. A new entity?

MADAM, Drs Sayami and Tagami (1984) report the case of 'a 15-year-old boy suffering from pruritic, corticoid-resistent, dapsone responsive, papulovesicles on the upper trunk and neck with granulocytic spongiosis', and suggest that this may constitute a new entity. They propose the term 'granulocitic spongiotic papulovesiculosis' for this condition.

We have had the opportunity to closely observe a patient with a mucocutaneous eruption whose skin lesions (Fig. 1) resemble those in the photograph of Drs Sayami and Tagami's patient.

Our patient, a 22-year-old female, was initially seen in August 1982 with a 2-week history of a non-pruritic afebrile vesicopustular eruption. She has been observed over a period of 33 months. The vesicopustules ranged in size from 0·5 to 1·5 mm and were symmetrically distributed on the face (particularly periorbital areas), neck, axilary and antecubital folds, umbilical and lateral abdominal areas

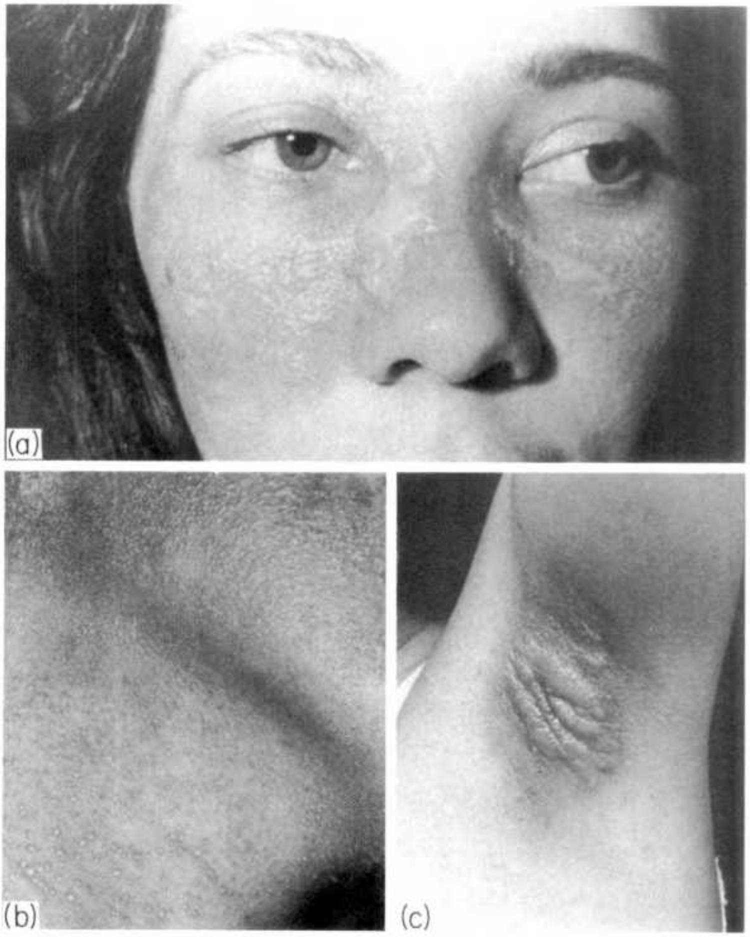

FIGURE 1. (a) Confluent vesicopustules forming large bullae on an erythematous base on the face. (b) Vesicopustules on the neck and face; early vesicles have a 'dew drop' appearance. (c) A cluster of vesicles on an erythematous base in the axilla.

ungueal bed, with severe involvement of the entire oral, pharyngeal, hypopharyngeal and vaginal mucous membranes, with erosions and denuded surfaces covered with a necrotic slough, sparing the lips, scalp, palms and plantae. The pain from oral and urogenital mucosal disease is severe, making eating and miction difficult. The patient has been admitted to hospital six times within the 33 months of observation, because of the extensive involvement of the mucous membranes which caused complications including dehydration and electrolyte imbalance. Eruptions of the crops of vesicopustules were preceded by headache, anxiety, dizziness, insomnia and intense local burning, and these were followed by the explosive appearance of the skin and mucous membrane lesions. Early vesicles had a 'dew drop' appearance, and arose on clinically normal skin or on erythematous bases; within a few hours the contents turned turbid, the vesicles never becoming umbilicated. Grouping of the vesicles occurred in the axilary and periorbital areas, and only in these areas the skin was erythematous with oedema. The eruption could be confined to the skin or be associated with mucous involvement. The patient experienced episodes of varying severity which could be localized or generalized, but the rash was monomorphous. Successive and subintrant crops appeared, persisted for 1–2 weeks and receded, sometimes leaving transient residual hyperpigmentation.

Medical history and other physical investigations were unremarkable. The family history was non-contributory.

Laboratory investigations showed full blood count, ESR, platelets, CRP, glucose, serum proteins, urea, creatinine, and urinalysis all within the normal reference ranges. ANA (on rat liver, Hep-2, and KB-cell), bacteriological and mycological investigations were negative. Microscopic examination of haematoxylin-eosin stained sections showed vesicopustules arising at sites of spongiosis and intracellular oedema, enlarging through reticular degeneration of epidermal cells. Neutrophilic infiltration was a striking feature, occurring from the upper dermis around the capillaries, through the epidermis, filling the vesicopustules, up to the stratum corneum (Fig. 2). Direct immunofluorescence studies were negative in July 1983 and positive with granular deposition of IgA at the dermo-epidermal junction in December 1984.

Treatment with rifampicin, tetracyclines, gentamicin, erythromycin, antihistamines, corticosteroids, azathioprine, methotrexate, zinc sulphate, thalidomide, ketoconazole and colchicine were ineffective. In December 1984, the patient was started on dapsone, 100 mg/day. Within 48 h she was free from lesions. The drug was discontinued on four occasions because of symptoms of intolerance including nausea, vomiting, headache, cyanosis and dyspnoea. These interruptions were followed by recurrences of the lesions which subsided promptly after resuming therapy with dapsone, 50 mg/day. Haematological evaluation revealed haemolytic anaemia, methaemoglobinaemia (35%), and sulphaemoglobinaemia (2%). Treatment with dapsone was withheld. We tried clofazimine, 100 mg/day, in view of its dapsone-like action in some neutrophilic diseases. The therapeutic response was similar to that with dapsone. Another crop of lesions emerged 4 days after discontinuing clofazimine, which subsided promptly after restarting the drug.

The most important features in this patient are the neutrophilic spongiosis comprising the fine sterile vesicopustular elements of the dermatosis, the monomorphic nature of the rash, the involvement of the skin and mucous membranes, the severe local burning sensation preceding the eruption, the absence of pruritus, the afebrile nature of the disease, and the striking as well as apparently exclusive responsivess to dapsone and clofazimine.

The differential diagnosis includes the neutrophilic spongiotic vesicopustular dermatoses such as dermatophytic infections, allergic and irritant contact dermatitis; sterile pustular dermatoses such as pustular psoriasis, pustulosis acuta generalisata (Tan, 1974), sterile eosinophilic pustulosis (Ofuji *et al.*, 1970; Orfanos & Sterry, 1978), subcorneal pustular dermatosis (SPD) (Sneddon & Wilkinson, 1956, 1979); and dermatitis herpetiformis (DH) The clinical and laboratory data rule out all these entities. In particular, IgA deposits at the dermo-epidermal interface—the suprabasal cleavage excludes DH and the absence of arcuate or annular configurations excludes SPD. Our patient resembles Drs Sayami and Tagami's patient as may be judged by the clinical appearance and the same therapeutic response to dapsone, but differs in other clinical and histological features.

The clinical and histological features observed in this patient are unique and distinct from those of the

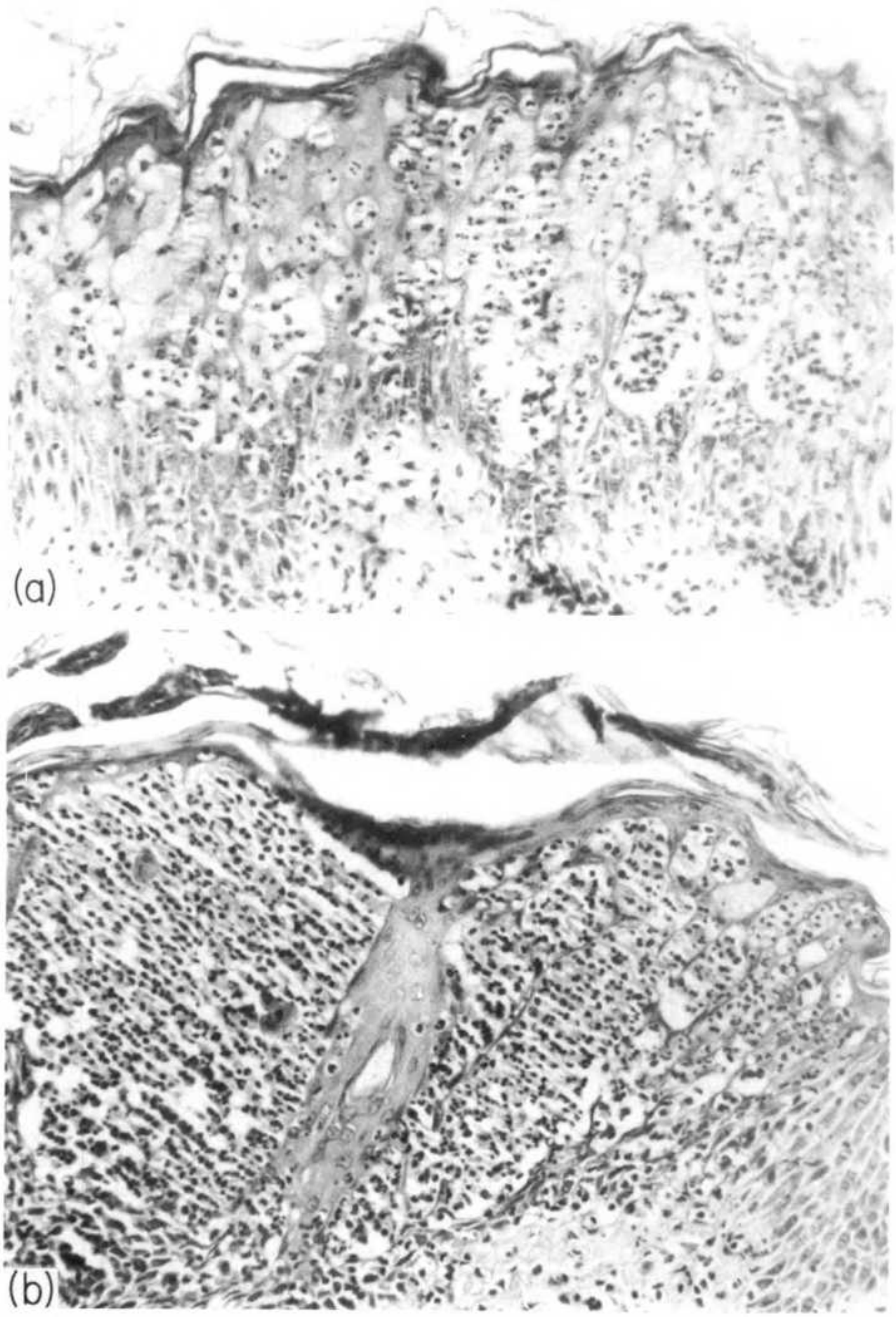

FIGURE 2. (a) Intraepidermal vesicles arise at sites of spongiosis and intracellular oedema. (b) From right to left; spongiosis, microvesicles filled with neutrophils, and a larger vesicopustule with a few acantholytic cells. Intense exocytosis of neutrophils is characteristic.

above mentioned dermatoses. To our knowledge there are no reports of similar cases in the literature. We suggest the term 'neutrophilic spongiosis' for this dermatosis.

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## Familial malignant atrophic papulosis (Degos' disease)—additional evidence for heredity and a benign course

Madam, We recently described six cases of malignant atrophic papulosis (MAP) occurring in one family, and suggested a genetic aetiology, (Kisch & Brunzeel, 1984). We have since studied another family with MAP. The proband, a 57-year-old woman, showed about 100 asymptomatic pea-sized pink papules, and lesions with a pink, telangiectatic rim and a porcelain-white centre. The lesions had started 1 year previously; there was no evidence of systemic involvement. A biopsy specimen showed a focal atrophic epidermis with hyperkeratosis, covering an oedematous mucin-rich dermis; no obliterated blood vessels could be detected. Examination of other members of the family revealed similar lesions in a 30-year-old daughter. This brings the number of families with MAP so far reported to four [our two families and the cases reported by Hall-Smith (1969) and Moulin *et al.* (1984)]. These observations strongly suggest genetic factors, possibly autosomal dominant inheritance with a high penetrance and a variable expressivity. Another possibility is the vertical transmission of a slow virus; however, inoculation tests of material from one of our patients and from a previously reported case (Moulin *et al.*, 1984) are negative so far. Another important conclusion is that the disease in a considerable number of patients appears to be limited to the skin and runs a benign course. Our previously reported patients still have no evidence of extracutaneous involvement.

We suggest that, in future cases, other family members should be examined and a potentially malignant course should not be over emphasized.

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## Rapid and simple macrophotography of the skin

Madam, Attempts have been made to improve the resolving power of the human eye for diagnostic purposes in dermatology, mainly by the use of the operating microscope (Fritsch & Pechlaner, 1981; Haas, Ernst & Stüttgen, 1984; MacKie, 1971). Although quite accurate (MacKie, 1971) this method suffers from the inconvenience of handling the bulky microscope and difficulties with focusing, as well as the rather high price of the instrument.

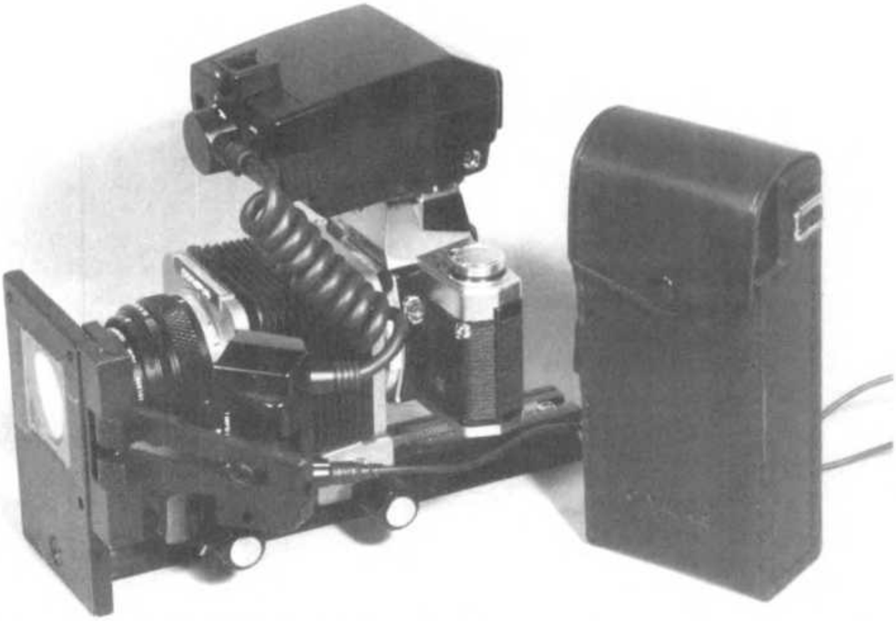

FIGURE 1. Apparatus for macrophotography of the skin consisting of camera, bellows, macrolens, footplate with glass window, flash unit and power pack.

We have developed a simple system based largely on parts of the Olympus OM camera system. The backbone of the apparatus is the bar with the bellows, equipped either with a 38 mm (*f* 2·8) or a 20 mm (*f* 2) macrolens. The camera body is a 35 mm SLR OM2 with an autoexposure electronic shutter. The bar carries a footplate made of 5 mm aluminium with a glass window with a diameter of about 35 mm along the optical axis. On one side the footplate carries an arm with the flash unit attached. The flash unit, equipped with a bright pilot light, is connected by a cable to the power control unit. The latter is fitted to the camera body by means of the flash shoe. The metering system of the camera automatically controls flash output, thus giving correct exposure under all conditions (Fig. 1).

Equipped with the 20 mm or 38 mm macrolens, the unit allows photographs to be taken with maximal magnifications of up to × 16 or × 6 7 respectively.

Photography of the skin with this apparatus is very simple. The footplate is put onto the skin with the pilot light on, either with or without immersion oil. Focusing and magnification are set by the knobs on the bellows. Once set, they can be kept fixed indefinitely by tightening the knobs. Photos are taken, as with all cameras, by pressing the shutter release. Any of the vast number of different 35 mm films available may be used, including the new instant films.

Our investigation of pigmented skin lesions (unpublished) has been done mainly with standard colour slide film, which allows easy secondary magnification by projecting the slides. Black and white films are also suitable.

The simple apparatus described greatly facilitates macrophotography of the skin surface and skin lesions. Apart from possibly aiding in the differential diagnosis of pigmented skin lesions and their macroscopic changes with time, the system might also be of value for the documentation of vascular lesions before and after treatment. Other possible applications, e.g. in microlymphangiography, remain to be developed.

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## Hyperdiploid cells in skin infiltrates

Madam, We have read with great interest the article by Ralfkiaer *et al.* (1985) on 'Single cell DNA measurements in benign cutaneous lymphoid infiltrates and in positive patch tests'. We would like to comment, however, on some of their results and on their interpretation.

From Tables 3 and 4 of Ralfkiaer's paper it is apparent that the DNA index in lymphoid cells infiltrating the skin is higher than in mononuclear cells from blood. This is not to say that the DNA content per cell is

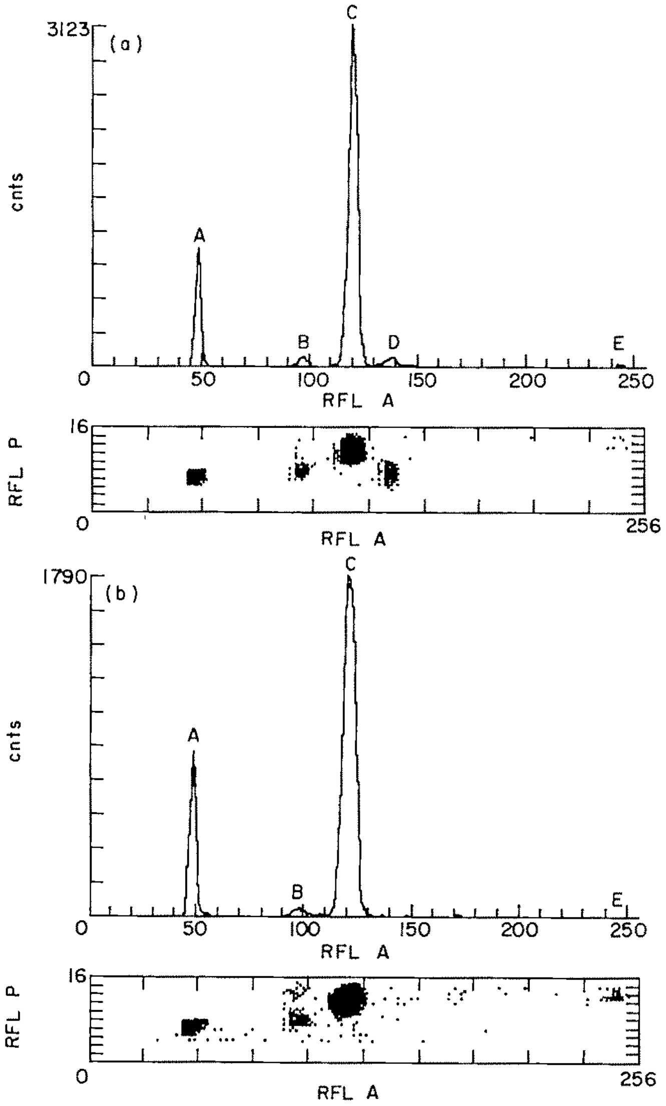

FIGURE 1. DNA-histograms (above) and two-dimensional scatterplot of peak fluorescence versus area (below) for mononuclear cells derived from blood. (a) Freshly suspended in Tris–EGTA buffer. (b) After 60 min additional incubation at 37°C A and B: internal standards; C: main population; D: 'hyperdiploid' population; E. doublets of C.

really different, but most probably that there are differences in stainability of the DNA, possibly caused by differences in the preparation procedure. Hence, the *biological* significance of that finding remains open. We think the authors would agree on that. Next then, in many of the samples the existence of an additional population, the so called 'hyperdiploid' peak, is hypothesized, this because of the right-skewed DNA histogram. This 'hyperdiploid' population is not always present and is also not consistent within a single individual (patients 3 and 4, Table 4).

The authors state 'that the hyperdiploid DNA histograms . . . are at least in part due to increased cellular

DNA' and that 'cells with propidium iodide fluorescence in the hyperdiploid region . . . . results from DNA replication and/or chromatin dispersion during cellular activation and proliferation'.

Firstly, we would like to comment on the use of the word 'hyperdiploid'. Hyperdiploidy is a cytogenetic term and means more *chromosomes* than the diploid number, where diploidy is defined as a doublet of the haploid number and euploidy as multiples of the haploid number. Abnormal sets of chromosomes have serious biological implications. In flow cytometry the amount of DNA per cell is measured. Diploid cells therefore should appear ideally in one channel, but in practice will appear as a normal distribution around a certain chosen channel number. Increase in DNA content during the cell cycle is reflected by the appearance of events in higher channel numbers (up to twice the channel numbers for cells in $G_2M$). Neither non-stochiometric staining with propidium iodide nor cells with more DNA due to the fact that they are cycling, justify the term hyperdiploid.

Secondly, we agree with the comment that there might be cycling cells in the population of the infiltrate cells. If the skewness of the right flank represents cells in S-phase, however, the estimate of the number of S-phase cells is incorrect since the fitting procedure used (zero-degree polynomial) excludes this population.

Thirdly, we would like to present some data from our own measurements on mononuclear cells from blood. The DNA index of mononuclear cells together with appropriate standards depends on the buffers used. We found indices of $1{\cdot}00 \pm 0{\cdot}02$, $0{\cdot}99 \pm 0{\cdot}02$, $0{\cdot}96 \pm 0{\cdot}01$ for the buffers Tris–EGTA, citrate and phosphate, respectively. This already indicates that the accessibility of DNA for the fluorochrome can easily be modified. Moreover, in the samples suspended in Tris–EGTA and in citrate, a separate 'hyperdiploid' cluster was often present that was not seen in the samples suspended in phosphate buffer. These subpopulations were morphologically distinct from the main cluster, indicated by the area and peak values of the fluorescence intensity profile (Ortho 50-H system, Fig. 1). Most interestingly, by incubating the suspension for 1 h at 37°C the 'hyperdiploid' cluster shifted back to 'normal' DNA values and a single perfectly symmetrical normal DNA distribution was obtained. All these observations are strongly in favour of the interpretation that the 'hyperdiploid' peak really represents a diploid population of cells with an altered chromatin structure.

These findings are extremely important since 'hyperdiploidy' in blood mononuclear cells in patients suspected to be suffering from T-cell lymphomas have been interpreted as 'diagnostically significant' (Lange Wantzin *et al.*, 1983). In conclusion we agree with the authors' remark that the potential application of this method in the discrimination between benign and malignant cutaneous lymphoid infiltrates still needs to be determined.

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R.M.DE GROOD
P.C.M.VAN DE KERKHOF

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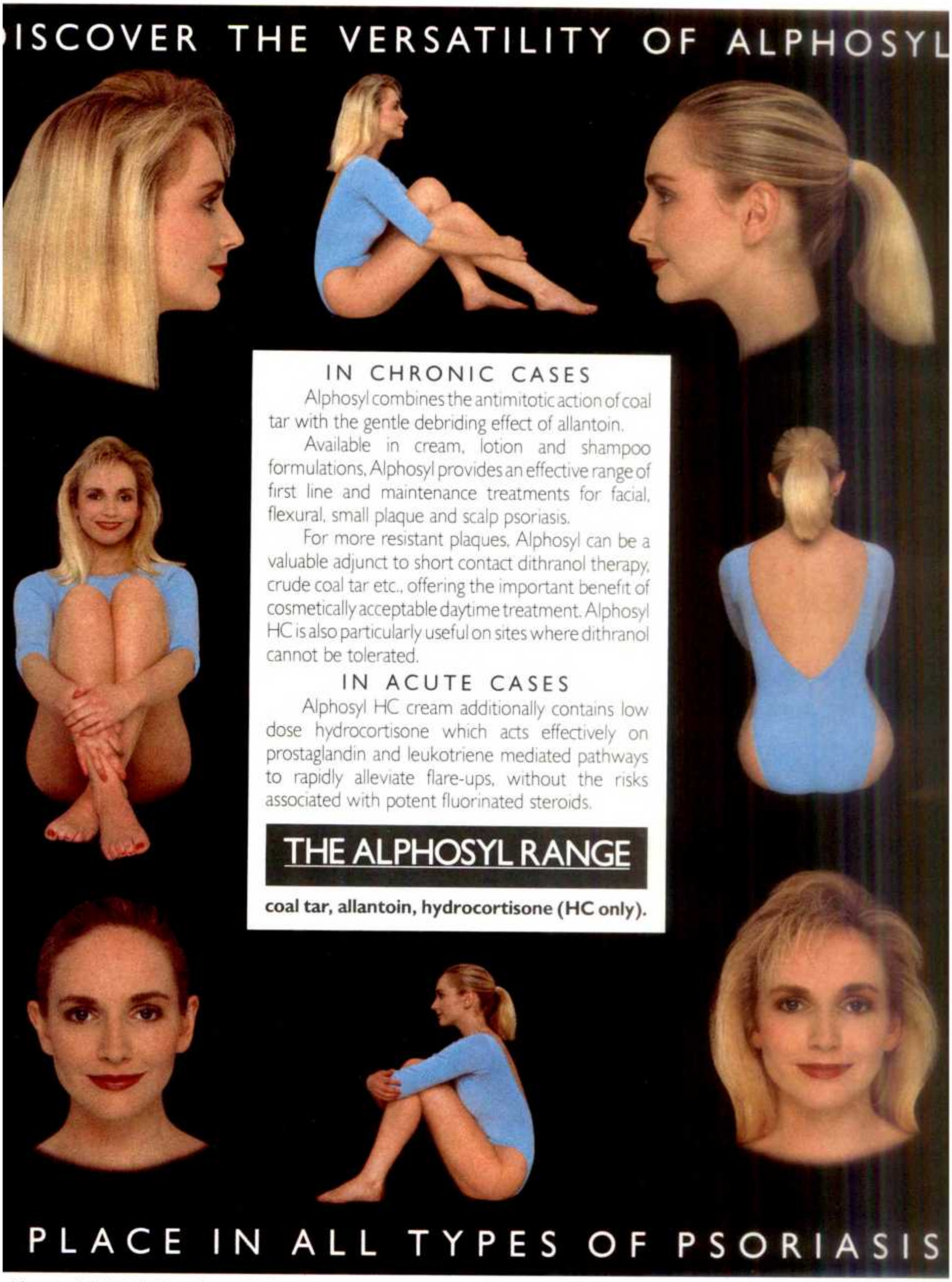

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| milligram(s) ($10^{-3}$g) | mg | second(s) | s | gravitational acceleration | *g* |
| microgram(s) ($10^{-6}$g) | μg | cubic millimetre(s) | mm$^3$ | micrometre(s) | μm |
| nanogram(s) ($10^{-9}$g) | ng | millimetre(s) | mm | per cent | |
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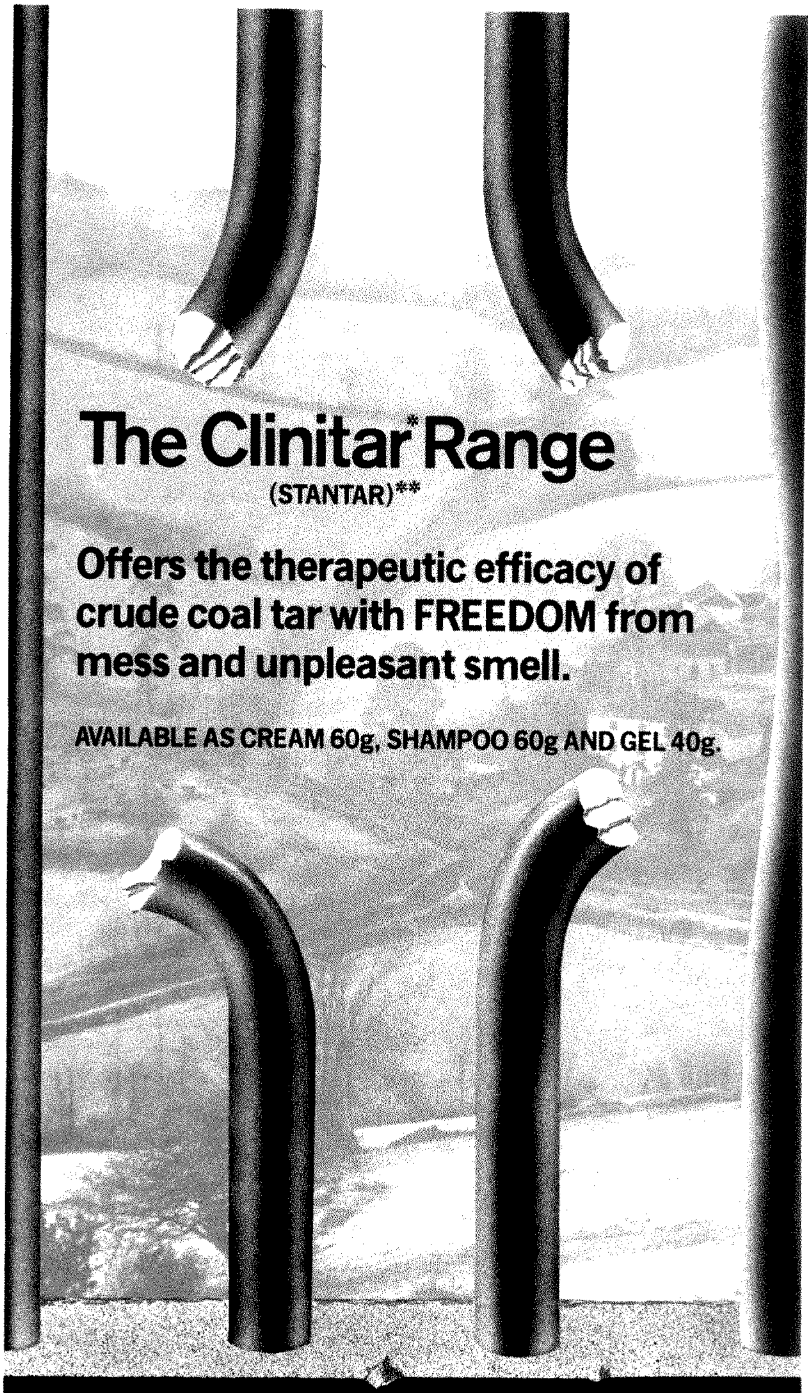

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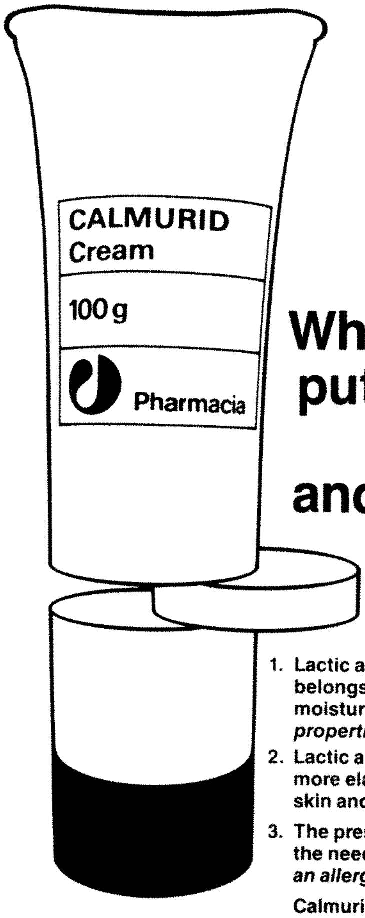

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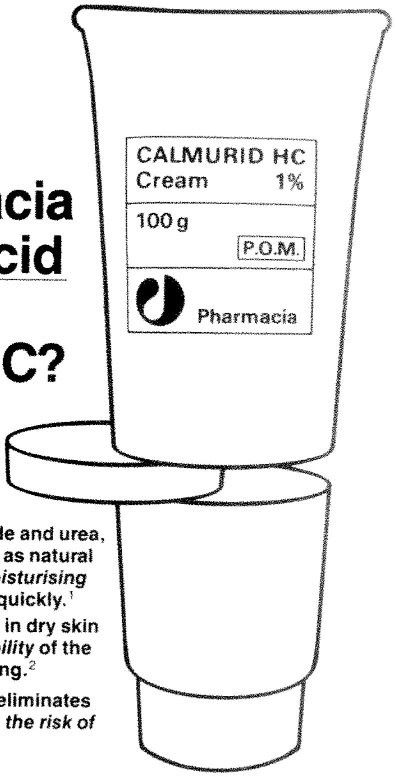

1. Lactic acid, together with sodium chloride and urea, belongs to a group of substances known as natural moisturising factors. It has *very good moisturising properties* and helps dry skin to recover quickly.[1]
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## Calmurid

**10% urea**

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**5% lactic acid in an emulsifying base**
**The natural way to treat**

**Dry Skin with Hyperkeratosis**

**Dry Eczema with Hyperkeratosis**

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**CALMURID.** ***Presentation*** Cream containing 10% urea. 5% lactic acid in an emulsifying base. ***Uses*** Hyperkeratosis, ichthyosis and other dry, rough, scaly skin conditions. ***Dosage and Administration*** Apply a thick layer twice daily after washing and drying. ***Contra-indications, warnings etc. Adverse Effects*** Calmurid can cause smarting temporarily when applied to raw or fissured areas. ***Pharmaceutical Precautions*** Store in a cool place but do not freeze. Do not put in alloy containers. ***Legal Category*** G.S.L. ***Package Quantities and Prices (May 1985)*** Tubes of 50g (£1.69). Tubes of 100g (£3.33). Tubes of 300g (£9.22). ***Further Information*** Calmurid is a pleasant cream which does not contain lanolin or preservatives. This results in a negligible risk of allergic reactions and thus good patient compliance. ***Product Licence Number*** 0009/5009.

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**Invited Editorial**

# Current understanding of the aetiology and clinical implications of cutaneous graft-versus-host disease

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Graft-versus-host disease (GVHD) occurs whenever lymphoid cells from an immunocompetent donor are introduced into a histo-incompatible recipient which is incapable of rejecting them. Cutaneous GVHD in man is most commonly seen as a complication of therapeutic bone marrow transplantation, but has also been reported to occur following maternofetal cell transfer in immunodeficient children and after transfusion of non-irradiated blood or blood products into immunodeficient subjects. Given that cutaneous GVHD is essentially seen only in a few specialized centres, why should this dermatological rarity continue to receive so much attention in the literature? The reason, of course, is that GVHD in its acute and chronic forms shares clinical, histological and immunological features with several important lymphocyte-mediated skin diseases (Saurat, 1981; James & Odom, 1983), and provides a unique biological model for the study of the immunopathology of epidermal-lymphocyte interactions (Breathnach & Katz, 1985a). Three recent papers report on immunohistochemical changes in the skin following therapeutic bone marrow transplantation in humans (Atkinson *et al.*, this issue; Dreno *et al.*, 1986; Lever *et al.*, this issue). This editorial will review briefly what is known about the pathological mechanisms involved in cutaneous GVHD and how such knowledge may afford insights into the pathogenesis of other lymphocyte-mediated skin diseases, as well as current information on the prevention, treatment and prognostic significance of GVHD following bone marrow transplantation.

## AETIOLOGY OF GVHD

Although it is generally accepted that the manifestations of GVHD are the result of donor T-lymphocyte responses to recipient transplantation antigens (Korngold & Sprent, 1978), the cellular mechanisms involved remain unclear. Inevitably, much of the available information has come from animal studies. Alloreactive cytotoxic T lymphocytes (CTL) seem to be neither necessary nor sufficient to induce acute lethal GVHD, which is thought to be mediated mainly by alloreactive donor T-suppressor cells which potentiate infection; the 'stimulatory' features of chronic GVHD, including production of autoantibodies characteristic of systemic lupus erythematosus, are thought to be mediated by donor alloreactive T-helper cells (*see* review by Breathnach & Katz, 1985a). These findings do not exclude a role for CTL in epithelial injury. The histology of human acute cutaneous GVHD is characterized by an upper dermal perivascular infiltrate, lymphoid exocytosis into the epidermis, and the presence of vacuolated dyskeratotic keratinocytes in the basal and spinous layers, often with adjacent lymphocytes (satellite cell necrosis) (Slavin & Santos, 1973; Sale *et al.*, 1977). This has led to the concept of the 'aggressor lymphocyte' as an effector cell in GVHD and other lymphocyte-mediated skin diseases. However, a similar histological appearance can result from X-irradiation and cytotoxic therapy alone (Sale *et al.*; 1977). The traditional view of the importance of cell-mediated lympholysis in skin diseases, including GVHD, has recently been called into question (Tyler *et al.*, 1984; Breathnach & Katz, 1985a; Atkinson *et al.*, this issue); some authors have proposed that cutaneous damage in GVHD may be mediated by natural killer cells rather than CTL (Snover, 1984). The evidence for the occurrence of CTL-mediated epidermal damage in GVHD and other lymphocyte-mediated skin diseases is in fact largely circumstantial. Haptenated murine epidermal cells can certainly act as targets for hapten-specific CTL *in vitro* (Tamaki *et al.*, 1981), and immunohistochemical studies have demonstrated the presence of intra-epidermal T cells of suppressor/cytotoxic phenotype in human GVHD (Lampert, *et al.*, 1982; Gomes *et al.*, 1982; Sloane *et al.*, 1984). The caveat remains, however, that the phenotype of a lymphoid cell defined immunohistochemically may not always accurately reflect its functional capacity. Perhaps the best evidence for an *in vivo* role for CTL in epidermal damage is the finding that cloned CTL injected intradermally into histo-incompatible mice produce immunologically specific destruction of allogeneic epidermis in a vigorous localized graft-vs-host reaction (Tyler *et al.*, 1984).

It is also not known exactly why the skin should be such a major target organ in GVHD, and which cells are the primary target for destruction. In addition to class I major histocompatibility complex (MHC) antigens, murine epidermal cells express certain unique skin-specific transplantation antigens such as Skn and Epa-1 which are capable of inducing allograft rejection, and which may be of relevance in regard to the first point (*see* review by Breathnach & Katz, 1985a). Homologous skin-specific antigens have not as yet been identified in humans. With regard to the second point, it has been suggested that stem cells in the basal layer of rete ridges, or their early progeny, may be specific targets, as a result either of osmotic fragility or of expression of early differentiation antigens (Sale *et al.*, 1985). An alternative viewpoint is that Langerhans cells (LC), which are the only cells in normal epidermis which express class II MHC (Ia, immune response-associated, HLA-DR) antigens, may be a particular target for destruction in cutaneous GVHD, since Ia antigen is a major stimulatory determinant in GVHD, and since LC have an important function in allograft rejection and are the critical stimulator cells in the *in vitro* allogeneic epidermal cell-lymphocyte reaction (*see* review by Breathnach & Katz, 1985a). Keratinocytes might then be damaged by an 'innocent bystander' effect as a result of lymphokine release (Snover, 1984). Immunohistochemical studies have shown that $Ia^+$ LC are decreased in GVHD (Lampert *et al.*, 1982; Gomes *et al.*, 1982; Suitters & Lampert, 1983; Sloane *et al.*, 1984; Murphy *et al.*, 1985), and an ultrastructural study has provided morphological evidence of lymphocyte-mediated damage to Langerhans cells (Gallucci *et al.*, 1979). These findings may be related in part to the effects of pre-transplant therapy, since X-irradiation alone decreases the number and antigen presenting capacity of $Ia^+$ Langerhans cells in rodents (Suitters & Lampert, 1983; Breathnach & Katz, 1985b), and there has been a lack of correlation between Langerhans cell number and the severity of histological changes of GVHD in some studies (Sloane *et al.*, 1984; Murphy *et al.*, 1985). Lever *et al.*, (this issue), however, found a reduction in epidermal Langerhans cells helpful in the early diagnosis of GVHD, and Perreault *et al.* (1984) noted a link between decreased Langerhans cell numbers and severity of acute GVHD. We found a progressive decrease in the number and antigen presenting capacity of $Ia^+$ Langerhans cells during the evolution of murine acute cutaneous GVHD which was considerably greater than that attributable to the effect of X-irradiation alone, a finding compatible with the interpretation that Ia$^+$ Langerhans cells are selectively lost in GVHD (Breathnach *et al.* 1986).

What conclusions can be drawn from these findings about pathological mechanisms underlying other lymphocyte-mediated skin diseases? It is tempting, although more than somewhat speculative, to suggest that in allergic contact dermatitis, mycosis fungoides (in both of which ultrastructural studies have shown lymphocytes adjacent to necrotic Langerhans cells (Silberberg, Baer & Rosenthal, 1976; Rowden *et al.*, 1979), lichen planus and lichenoid drug eruptions, Langerhans cells which have processed and expressed on their cell surface foreign (viral, drug, or altered self) antigens form targets for specific CTL, resulting in foci of epidermal damage.

Keratinocytes do not express Ia antigen in normal skin, but have been reported to express this antigen in a variety of lymphocyte-mediated skin diseases, including allergic contact dermatitis, the tuberculin reaction, mycosis fungoides/cutaneous T cell lymphoma, lichen planus, lupus erythematosus and allograft rejection, in addition to GVHD (Lampert, Suitters & Chisholm, 1981; Mason, Dallman & Barclay, 1981; Breathnach & Katz, 1985a). We and others have shown that keratinocyte Ia antigen in these conditions is synthesized by the keratinocytes themselves (Breathnach & Katz, 1983; Volc-Platzer *et al.*, 1984); Ia synthesis is thought to occur in response to gamma-interferon secreted by activated T cells. Induction of Ia expression on a variety of other normally Ia-negative cell types, including thyroid follicular epithelial cells in autoimmune thyroiditis, has resulted in acquisition of antigen presenting capacity (Londei *et al.*, 1984; Breathnach & Katz, 1985a) and has been suggested as an important mechanism underlying the development of autoimmunity. Therefore, if it could be shown that Ia$^+$ keratinocytes were able to present antigen directly to T cells, leading to greatly enhanced cutaneous immune responses and disease chronicity, a major advance in our understanding of the pathogenesis of lymphocyte-mediated skin diseases would be achieved. We have as yet been unable to demonstrate any antigen presenting capacity of Ia$^+$ keratinocytes in murine GVHD (Breathnach *et al.*, 1986). However, the experimental design of our study necessitated lethal irradiation of the mice, which may have abrogated any potential antigen presenting function of Ia$^+$ keratinocytes. An alternative consequence of keratinocyte Ia expression may be that of increased keratinocyte destruction by CTL, since the CTL response to allogeneic cells is greatly enhanced when class I MHC and class II MHC/Ia/HLA-DR antigenic stimuli are provided.

## PREVENTION AND PROGNOSTIC SIGNIFICANCE OF GVHD

The incidence of GVHD varies between 35% and 75% in different series, and the overall mortality of acute GVHD ranges between 7% for patients with grade 1 disease to 100% in those with grade 4 changes; the mortality at 5-year follow-up in patients with severe chronic GVHD may be as high as 30% (Wick *et al.*, 1983). The risk of GVHD following HLA-matched bone marrow transplantation is increased in patients with acute lymphocytic leukaemia, in sex-mismatched donor-recipient pairs, and in patients over 24 years old. Established acute GVHD is difficult to reverse, although it may respond to high dose corticosteroids, either alone or combined with methotrexate or antilymphocyte globulin. The prevention and management of GVHD has recently been extensively reviewed (Neudorf, *et al.*, 1984; Harper, *et al.*, 1984; Editorial, 1984; Denman, 1985). Prophylactic regimes have most commonly involved the use of methotrexate, prednisone, anti-thymocyte globulin, or cyclosporin A. Cyclosporin A therapy has proved very effective in decreasing the severity but not the incidence of acute GVHD.

Cyclosporin A therapy is expensive, nephrotoxic at high dosage, and potentially oncogenic. Therefore, attention is being focused on methods for selectively removing mature T lymphocytes, which initiate GVHD, from donor marrow while preserving pluripotential stem cells; the T lymphocyte progeny of such grafts which differentiate in the host environment are thought to be tolerized to host antigens. Techniques currently being assessed include treatment of donor marrow with anti-T cell monoclonal antibodies either singly, in combination, or linked to the toxin ricin. Knowledge gained from manipulating lymphoid cell subsets in this manner for the prophylaxis of GVHD may eventually prove of wider significance in the management of patients with connective tissue diseases or cutaneous T cell lymphoma.

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***Clinical and Laboratory Investigations***

# Mononuclear cell subpopulations in the skin defined by monoclonal antibodies after HLA-identical sibling marrow transplantation

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## SUMMARY

Mononuclear cell subpopulations present in the skin of 36 recipients of HLA-identical sibling marrow transplants were defined by immunoperoxidase using a battery of monoclonal antibodies to cell surface differentiation antigens. The T4-positive (T4+) (helper-inducer T cells), T8+ (cytotoxic-suppressor T cells) and the T6+ (Langerhans cells) decreased in number early post transplant and returned towards normal numbers from day 42 onwards. There was no evidence that either the T4+ or the T8+ subset was involved in cell-to-cell contact damage in acute graft-versus-host disease (GVHD). The paucity of lymphoid cell infiltration of the epidermis in acute GVHD suggested the possibility of a soluble factor being responsible for basal layer damage. In patients with chronic GVHD there was no evidence of T4+ lymphocyte involvement, but T8+ lymphocytes were present in increased numbers, suggesting a role for the T8+ population in the skin lesions of chronic GVHD, or possibly a reflection of the pattern of T4+ and T8+ cell reconstitution in the blood post-transplant. Finally, our study provided no evidence that B1+ (B cells), Leu 7+ (natural killer cells), OKM1+ (histiocytes) or OKT10+ cells were involved in cell-to-cell contact damage in either acute or chronic GVHD.

Incubation of murine bone marrow with anti-Thy 1 antibody prior to its infusion into an irradiated histo-incompatible recipient can result in a marked reduction in the incidence and severity of graft-versus-host disease (GVHD) (Korngold & Sprent, 1978; Onoe, Fernandes & Good, 1980; Vallera *et al.*, 1982). There therefore appears little doubt that T cells are the trigger mechanism for GVHD. There is, however, a paucity of data on the mechanisms of target organ damage in this disease. In an attempt to determine the cell population or populations important in the development of skin GVHD in man, we used a battery of monoclonal antibodies to

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determine the phenotype of mononuclear cell subpopulations infiltrating the skin during acute and chronic GVHD in 36 recipients of HLA-identical sibling marrow transplants. We performed sequential biopsies on 24 patients during the first 3 months post-transplant and single biopsies in 22 long-term survivors, 10 of whom had also been monitored sequentially.

## METHODS

### *Patients*

After conditioning with either cyclophosphamide alone or cyclophosphamide and total body irradiation, 36 patients were given HLA-identical sibling marrow transplants as treatment for haematological malignancy (32 patients) or severe aplastic anaemia (four patients). Our therapeutic protocol for marrow transplantation has been previously described (Biggs *et al.*, 1983). Patients received either cyclosporin (CSP) (29 patients) or methotrexate (MTX) (seven patients) as immunosuppression to minimize graft-versus-host disease post-transplant. The day of transplant was designated day 0. The clinical and histological diagnosis of acute (Glucksberg *et al.*, 1974; Lerner *et al.*, 1974) and chronic (Sullivan *et al.*, 1981; Shulman *et al.*, 1980) GVHD have been previously described. All patients were treated on protocols approved by the Ethics Review Committee of St. Vincent's Hospital.

### *Skin biopsies*

Three millimetre punch skin biopsies from the posterior iliac crest area were taken sequentially pre-transplant and on days 14, 21, 28, 42, 63 and 84 in 24 patients at the time of diagnostic bone marrow aspirations. In addition, whenever a skin rash occurred during the first 3 months in these patients a biopsy of the rash was taken. A total of 22 patients surviving 6 months or longer post-transplant, 10 of whom had been biopsied sequentially during the first 3 months, had skin biopsies performed between 6 and 24 months post-transplant. Fourteen of these patients had clinical evidence of chronic GVHD and eight did not. All biopsies were placed in sterile gauze soaked in 0·9% sodium chloride solution, and were bisected upon arrival in the histopathology laboratory. One half was placed in formalin for haematoxylin and eosin paraffin section processing, and the other was snap-frozen for subsequent immunoperoxidase processing. Biopsies taken from the posterior iliac crest area of 20 marrow donors at the time of marrow donation were used to evaluate lymphoid cell subsets in normal skin.

### *Reagents*

The monoclonal antibodies used in this study are described in Table 1. Normal horse serum, biotinylated horse anti-mouse IgG immunoglobulin, and avidin and biotin peroxidase conjugate were purchased from Vector Laboratories, Burlingame, CA, U.S.A. in the form of Vectastain ABC kit. Tris-phosphate buffer solution was used in the preparation of all solutions and in the washing of sections. Tris (hydroxymethyl)-aminomethane 0·05 M in phosphate buffered saline solution pH 7·3 (Dulbecco) was adjusted to 7·6 with 0·1M HCl and brought up to 1000 ml with phosphate buffered saline. The peroxidase visualization reagent was 3·3′-diaminobenzidine tetra-hydrochloride (DAB) (Sigma, St. Louis, MO, U.S.A.) (15 mg) in Tris-phosphate buffered saline (50 ml). Hydrogen peroxide was used as a 1% aqueous solution prepared freshly from a 30% solution of guaranteed strength.

TABLE 1. Monoclonal antibodies used

| Monoclonal antibody | Cell population defined in this study* | Source | Reference |
|---|---|---|---|
| T4 | Helper-inducer T cells | Coulter Electronics, Hialeah, Florida | Reinherz *et al.* (1981) |
| OKT8 | Cytotoxic-suppressor T cells | Ortho Pharmaceutical Corporation/Ethnor Pty Ltd, Raritan, New Jersey and Sydney, Australia | Reinherz *et al.* (1980) |
| OKT6 | Langerhans cells (& mature thymocyte) | Ortho/Ethnor | Murphy *et al.* (1981) |
| B1 | B cells | Coulter | Stashenko *et al.* (1980) |
| αLeu 7 | Natural killer cells and subset of cytotoxic-suppressor cells. | Becton Dickinson, Mountain View, California | Abo & Balch (1981) |
| OKM1 | Monocytes, null cells (& granulocytes) | Ortho/Ethnor | Breard *et al.* (1980) |
| OKT10 | Activated T & B cells (thymocytes and haemopoietic precursors) | Ortho/Ethnor | Cotner *et al.* (1981) |
| αHLA-DR | Ia-like antigen bearing cells. | Becton Dickinson | Lampson & Levy (1980) |

* Populations in parentheses may also be identified by the respective antibodies.

### *Immunoperoxidase staining*

Skin punch biopsies were embedded in OCT compound (Tissue Tek, Naperville, IL, U.S.A.), snap frozen on pre-frozen OCT support in dry ice, cut at 6–8 μm in a cryostat at −22°C, collected on gelatin subbed slides, fixed immediately in acetone-ethyl alcohol mixture 2:1 at −10°C for 5 min and deposited in Tris–phosphate buffer (0·1 M) at pH 7·6 at room temperature for 5 min. Endogenously-occurring peroxidase was then blocked by a 3% aqueous solution of hydrogen peroxide for 5 min.

After thorough washing in the Tris–phosphate buffer for 15 min, normal horse serum (1:50) was applied for 5 min in a wet chamber with continuous agitation and, without washing, the primary murine monoclonal antibody was applied for 30 min at appropriate dilution. After multiple washes in the buffer for 5 min, biotinylated horse anti-mouse IgG immunoglobulin was applied for 20 min and, after further washes in buffer for 5 min, freshly prepared avidin-biotin-peroxidase complex (1:50) was applied for 30 min. The sections were then thoroughly washed in buffer and a modification of the peroxidase histochemical reaction was performed. The sections were first pre-incubated with DAB solution for 1 min; after the addition of 1 ml of hydrogen peroxide solution the incubation was continued for another 5 min. After a thorough wash, melanin pigment was differentiated by 0·5% methylene blue at pH 4·0 for 2 min and nuclei by Mayer's haematoxylin for 20 s. The preparations were then dehydrated, cleared in xylol and mounted in Permount.

The sensitivity and specificity of each of the monoclonal antibodies used in the study was

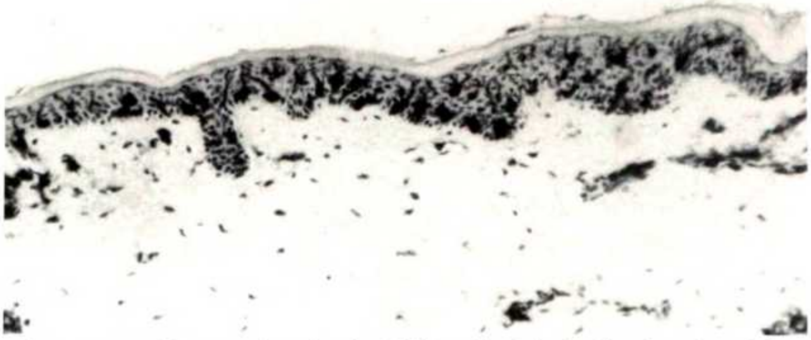

FIGURE 1. Immunoperoxidase reaction using the OKT6 antibody in the skin of a patient with acute graft-versus-host disease, day 42 post-transplant; haematoxylin counterstain. The dendritic processes present on most (but not all) of the positively-staining Langerhans cells are clearly seen (original × 168).

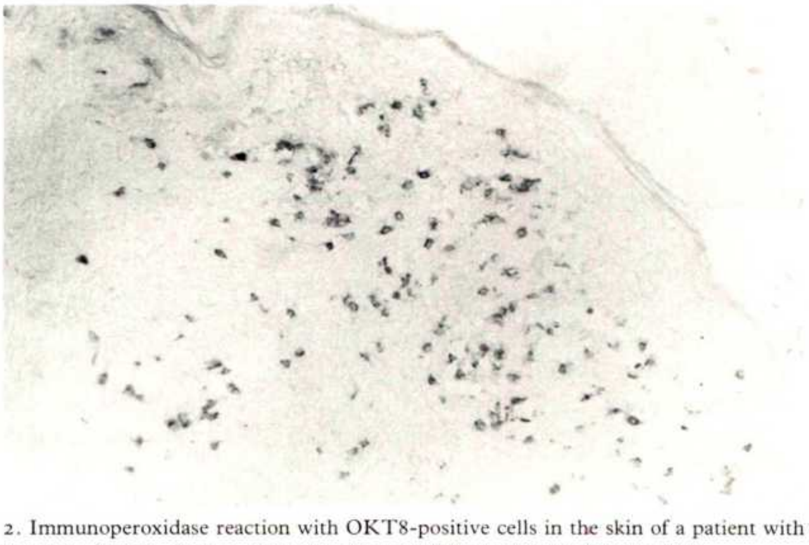

FIGURE 2. Immunoperoxidase reaction with OKT8-positive cells in the skin of a patient with chronic graft-versus-host disease, day 270 post-transplant; nuclei unstained (original × 168).

initially tested on normal lymphoid tissue (lymph node, spleen, thymus), and this was repeated with each new sample of antibody purchased. A section from each specimen was always processed as a negative control by omitting the monoclonal antibody from the normal sequence of immunoreagents. An example of OKT6-positive Langerhans cells is shown in Fig. 1, and of OKT8-positive cytotoxic-suppressor T cells in Fig. 2.

### *Evaluation of results*

Each section was read by one observer (VM) without knowledge of the clinical condition of the respective patient. The number of positively staining mononuclear cells present in the immunoperoxidase sections was evaluated as follows. No cells (−), no cells present on section; occasional cells (±), 1–2 cells on section; small numbers of cells (+), 1–2 cells present on more than 50% of high power fields; moderate numbers of cells (+ +), more than 2 cells present on more than 50%, but less than 100% of high power fields; large numbers of cells (+ + +), more than 2 cells present on 100% of high power fields.

### *Statistical analysis*

For the purposes of statistical analysis, the distribution of mononuclear cells in normal skin was compared with that in patient skin by chi squared analysis, after combining the proportion of cells in the categories 'no cells' and 'occasional cells' in one group, and the proportion of cells in the categories 'small', 'moderate' and 'large numbers' in a second group.

The two-tailed Z-test was used to make a further comparison between normal values and patient values at each time point post-transplant for the proportion of those individuals with 'no cells' and for the proportion of those individuals with 'occasional cells', whenever the number in each sample was sufficient.

### *Circulating mononuclear cells*

Since the nature of mononuclear cells present in the skin must to some extent depend on that of the mononuclear cells circulating in the blood, we also quantitated the number of circulating T4+ and T8+ T lymphocytes. The total white cell count was obtained on a Coulter S+ counter (Coulter Electronics, Sydney, NSW, Australia). The proportion of lymphocytes was obtained by routine Giemsa staining of the blood film. Heparinized peripheral blood was diluted 1:1 with phosphate buffered saline and centrifuged on a Ficoll–Hypaque gradient (Pharmacia, Sydney, Australia). Mononuclear cells were taken from the interface and incubated for 30 min at 4°C with fluorescein-conjugated OKT4 (Ortho Pharmaceutical Corp, Raritan, NJ, U.S.A.) or OKT8 (Ortho) monoclonal antibodies. The proportion of positively staining cells was enumerated using an Epics V flow cytometer (Coulter). The absolute number of T4+ or T8+ lymphocytes was then derived from the absolute lymphocyte count.

## RESULTS

### *Mononuclear cell subpopulations in normal skin*

The frequency of the various mononuclear cell subpopulations in normal skin is shown in Table 2. In the epidermis the majority of normal persons had no detectable T4+ or T8+ T cells, while a minority had occasional cells or small numbers of cells of both types present. The number of T4+ and T8+ cells was approximately equal. In contrast, almost all individuals had moderate or large numbers of OKT6+ Langerhans cells. No OKT10+, B1+ or Leu 7+ cells were noted and only one of 20 (5%) normal persons had an occasional OKM1+ cell present.

In the dermis, T4+ and T8+ T cells were commoner than in the epidermis and again the number of each subpopulation were similar. OKT6+ Langerhans cells were present in most individuals, but were less frequent than in the epidermis. No B1+ cells were noted, but occasional OKT10+ and Leu 7+ cells were found in a minority of persons, while a majority had small or moderate numbers of OKM1+ histiocytes.

### *Mononuclear cell subpopulations in the skin of marrow transplant recipients during the first three months post-transplant*

Sequential studies in 24 patients biopsied during the first three months post-transplant showed

TABLE 2. Mononuclear cell subpopulations in the skin of normal persons ($n=20$)

| | Frequency of cells* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Epidermis | | | | | Dermis | | | | |
| Cell population | No cells | Occasional cells | Small nos | Moderate nos | Large nos | No cells | Occasional cells | Small nos | Moderate nos | Large nos |
| T4+ | 65 | 25 | 10 | 0 | 0 | 35 | 0 | 50 | 15 | 0 |
| OKT8+ | 60 | 30 | 10 | 0 | 0 | 0 | 30 | 60 | 10 | 0 |
| OKT6+ | 0 | 5 | 0 | 75 | 20 | 15 | 5 | 40 | 40 | 0 |
| OKT10+ | 100 | 0 | 0 | 0 | 0 | 85 | 15 | 0 | 0 | 0 |
| B1+ | 100 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |
| Leu 7+ | 100 | 0 | 0 | 0 | 0 | 70 | 25 | 5 | 0 | 0 |
| OKM1+ | 95 | 5 | 0 | 0 | 0 | 5 | 0 | 56 | 39 | 0 |

* Values represent proportion (%) of individuals tested.

TABLE 3. Helper-inducer (T4+) and cytotoxic-suppressor (OKT8+) T cells in the epidermis during the first 3 months after HLA-identical sibling marrow transplantation

| | T4+* | | | | | T8+ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group | No cells | Occasional cells | Small nos | Moderate nos | Large nos | No cells | Occasional cells | Small nos | Moderate nos | Large nos |
| Normal donor | 65 | 25 | 10 | 0 | 0 | 60 | 30 | 10 | 0 | 0 |
| Patients Day+14 | 82 | $\boxed{6}$ | 6 | 6 | 0 | 83 | 11 | 6 | 0 | 0 |
| Day+21 | $\boxed{94}$ | $\boxed{6}$ | 0 | 0 | 0 | 82 | 18 | 0 | 0 | 0 |
| Day+28 | $\boxed{91}$ | 9 | 0 | 0 | 0 | 82 | 0 | 9 | 9 | 0 |
| Day+42 | 78 | 22 | 0 | 0 | 0 | 42 | 42 | 11 | 5 | 0 |
| Day+63 | 85 | 15 | 0 | 0 | 0 | 50† | 21† | 29† | 0† | 0† |
| Day+84 | 70 | 13 | 17 | 0 | 0 | 44† | 26† | 26† | 4† | 0† |

* Values represent proportion (%) of individuals tested.
Boxed values different from normal by two-tailed Z test ($P=0{\cdot}1$ to $<0{\cdot}005$)
† Distribution of cells different from normal by chi squared analysis, ($P<0{\cdot}005$).

a fall in the number of epidermal T4+ cells (Table 3) and T6+ cells (Table 5) early post-transplant. A similar downward trend in T8+ cell numbers was not statistically significant. The T6+ population returned towards normal from day 42 onwards (Table 5). The T4+ population showed only slight evidence of returning towards normal over this time period, and thus by day 84, T8+ cells were commoner in the epidermis than T4+ cells.

In the dermis (Tables 4 and 5) a similar pattern was noted although changes in the T8+ population were slight. It was clear from these sequential studies that the result of the interpretation of mononuclear cell subset numbers in the skin is dependent on the time post-transplant at which the biopsy is taken.

The predominant morphological damage in skin during acute GVHD occurs in the basal layer of the epidermis (Table 6). Twenty patients were biopsied at the time of acute GVHD of the skin (median day of biopsy, day 14) and the majority had no detectable T4+ (83%) or T8+ (90%) T cells present in the epidermis (Table 6). Additionally, B1+ B cells, Leu 7+ natural killer cells, and OKM1+ histiocytes were not detected in the epidermis of any patient with acute GVHD. One patient had small numbers, and one moderate numbers, of OKT10+ cells in the epidermis. These cells are not seen in normal skin. T6+ Langerhans cells were markedly reduced in the epidermis. Apart from a similar reduction of T6+ cells in the dermis and a reduction of OKM1+ histiocytes, the subset numbers in the dermis were little different from normal. We suggest from these findings that neither the T4+ nor the T8+ T cell subpopulation causes cell-to-cell contact damage in acute GVHD of the skin.

### *Mononuclear cell subpopulations in the skin of long-term survivors of allogeneic marrow transplants*

Twenty-two long-term survivors were biopsied between 6 and 66 (median 12) months post-transplant (Table 7). Fourteen of these had chronic GVHD and eight did not. In the epidermis the frequency of T4+ cells was similar to normal in both patient groups, but T8+ cells appeared more frequently, especially in those with chronic GVHD, of whom 50% had small or moderate numbers of T8+ cells in contrast to 10% of normal persons. There was a normal frequency of epidermal Langerhans cells in both patient groups. A small number of patients in both groups had occasional epidermal Leu 7+ cells, a finding not present in normal epidermis. No B1+ B cells, OKT10+ cells or OKM1+ cells were present in the epidermis of patients in either group. We conclude from these findings that neither T4+ T cells nor B1+ B cells, Leu 7+ cells, OKT10+ cells or OKM1+ cells are involved in cell-to-cell contact damage in the epithelium in chronic GVHD. The findings are consistent with a role for the T8+ lymphocyte subpopulation in the epithelial lesion of chronic GVHD.

In the dermis, T4+ cells were detectable less often than normal in patients both with and without chronic GVHD; T8+ cell frequency was not markedly different from normal. OKM1+ histiocytes were reduced in frequency in both patient groups. A striking difference between the two patient groups was a marked reduction in T6+ Langerhans cells in those patients with chronic GVHD (79% had none to occasional cells), while those without chronic GVHD showed a normal frequency (76% had small to moderate numbers). The frequency of B1+ B cells, Leu 7+ cells and OKT10+ cells was not different from normal in either patient group. We conclude from these findings that neither the T4+ nor the T8+ T cell subpopulations nor B1+ B cells, Leu 7+ cells nor OKT10+ cells are involved in direct cell damage to the dermis in chronic GVHD.

TABLE 4. Helper-inducer (T4+) T cells and cytotoxic-suppressor (OKT8+) T cells in the dermis during the first 3 months after HLA-identical sibling marrow transplantation

| | T4+* | | | | | OKT8+ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group | No cells | Occasional cells | Small nos | Moderate nos | Large nos | No cells | Occasional cells | Small nos | Moderate nos | Large nos |
| Normal Donors | 35 | 0 | 50 | 15 | 0 | 0 | 30 | 60 | 10 | 0 |
| Patients Day + 14 | 48† | $\boxed{12}$ † | 24† | 12† | 0† | $\boxed{11}$ | 11 | 61 | 10 | 0 |
| Day + 21 | 38† | $\boxed{31}$ † | 25† | 6† | 0† | $\boxed{12}$ | 24 | 46 | 17 | 0 |
| Day + 28 | 64† | 9† | 18† | 9† | 0† | 17 | 17 | 58 | 8 | 0 |
| Day + 42 | 21 | $\boxed{26}$ | 47 | 6 | 0 | 5 | 15 | 40 | 35 | 5 |
| Day + 63 | 23 | $\boxed{15}$ | 47 | 15 | 0 | 0† | $\boxed{0}$ † | 84† | 8† | 8† |
| Day + 84 | 17 | $\boxed{17}$ | 49 | 17 | 0 | 9 | $\boxed{4}$ | 48 | 30 | 9 |

* Values represent proportion (%) of individuals tested.

Boxed values different from normal by two-tailed Z test, ($P = 0·1$ to $< 0·005$).

† Distribution of cells different from normal by chi squared analysis, ($P < 0·015$ to $< 0·005$).

TABLE 5. Langerhans cells (OKT6+) present in the skin during the first 3 months after HLA-identical sibling marrow transplantation

| | Epidermis* | | | | | Dermis | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group | No cells | Occasional cells | Small nos | Moderate nos | Large nos | No cells | Occasional cells | Small nos | Moderate nos | Large nos |
| Normal Donors | 0 | 5 | 0 | 75 | 20 | 15 | 5 | 40 | 40 | 0 |
| Patients Day + 14 | $\boxed{47}$ † | $\boxed{29}$ † | 18† | 6† | 0† | $\boxed{84}$ † | 11† | 5† | 0† | 0† |
| Day + 21 | $\boxed{52}$ † | 18† | 24† | 6† | 0† | $\boxed{82}$ † | 6† | 12† | 0† | 0† |
| Day + 28 | $\boxed{58}$ † | 17† | 17† | 8† | 0† | $\boxed{92}$ † | 8† | 0† | 0† | 0† |
| Day + 42 | 10† | 5† | 30† | 40† | 15† | 35† | 25† | 25† | 15† | 0† |
| Day + 63 | 7 | 0 | 7 | 50 | 36 | 43† | 21† | 21† | 15† | 0† |
| Day + 84 | 4 | 0 | 22 | 48 | 22 | 29† | $\boxed{42}$ † | 24† | 5† | 0† |

* Values represent proportion (%) of individuals tested.

Boxed values different from normal by two-tailed Z test, ($P = 0·006$ to $< 0·0001$).

† Distribution of cells different from normal by chi squared analysis, ($P < 0·05$ to $< 0·005$).

TABLE 6. Mononuclear cell subpopulations in the skin of allogeneic marrow transplant recipients biopsied at the time of acute graft-versus-host disease of the skin ($n = 20$)*

| | Frequency of cells stained with appropriate antibody† | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Epidermis | | | | | Dermis | | | | |
| Antibody | No cells | Occasional cells | Small nos | Moderate nos | Large nos | No cells | Occasional cells | Small nos | Moderate nos | Large nos |
| T4+ | 83 | 11 | 6 | 0 | 0 | 39 | 11 | 28 | 22 | 0 |
| OKT8+ | 90 | 5 | 0 | 5 | 0 | 5 | 11 | 68 | 16 | 0 |
| OKT6+ | 53 | 16 | 26 | 5 | 0 | 80 | 15 | 5 | 0 | 0 |
| OKT10+ | 90 | 0 | 5 | 5 | 0 | 80 | 10 | 5 | 5 | 0 |
| B1+ | 100 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |
| Leu 7+ | 100 | 0 | 0 | 0 | 0 | 82 | 18 | 0 | 0 | 0 |
| OKM1+ | 100 | 0 | 0 | 0 | 0 | 39 | 0 | 28 | 33 | 0 |

* Skin biopsies performed at day 14 (median) (range 7–21) post-transplant.

† Values represent proportion (%) of individuals tested.

TABLE 7. Mononuclear cell subpopulations in the skin of long-term survivors of allogeneic bone marrow transplants, with and without chronic GVHD

| Cell population | Frequency of cells* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Epidermis | | | | | Dermis | | | | |
| | No cells | Occasional cells | Small nos | Moderate nos | Large nos | No cells | Occasional cells | Small nos | Moderate nos | Large nos |
| Without chronic GVHD ($n = 8$) | | | | | | | | | | |
| OKT4+ | 75 | 12·5 | 12·5 | 0 | 0 | 29 | 29 | 29 | 13 | 0 |
| OKT8+ | 38 | 62 | 0 | 0 | 0 | 12 | 12 | 38 | 38 | 0 |
| OKT6+ | 12 | 0 | 0 | 50 | 38 | 12 | 12 | 38 | 38 | 0 |
| OKT10+ | 100 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |
| B1+ | 100 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |
| Leu 7+ | 80 | 20 | 0 | 0 | 0 | 80 | 20 | 0 | 0 | 0 |
| OKM1+ | 100 | 0 | 0 | 0 | 0 | 57 | 0 | 14 | 29 | 0 |
| With chronic GVHD ($n = 14$) | | | | | | | | | | |
| T4+ | 79 | 7 | 14 | 0 | 0 | 58 | 14 | 21 | 7 | 0 |
| OKT8+ | 29 | 21 | 29 | 21 | 0 | 14 | 7 | 44 | 21 | 14 |
| OKT6+ | 0 | 0 | 14 | 72 | 14 | 44 | 35 | 14 | 7 | 0 |
| OKT10+ | 100 | 0 | 0 | 0 | 0 | 86 | 14 | 0 | 0 | 0 |
| B1+ | 100 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |
| Leu 7+ | 91 | 9 | 0 | 0 | 0 | 83 | 17 | 0 | 0 | 0 |
| OKM1+ | 100 | 0 | 0 | 0 | 0 | 36 | 0 | 57 | 7 | 0 |

* Values represent proportion (%) of individuals tested.

Boxed values indicate that distribution of cells is significantly different from normal, ($P < 0·025$ to $P < 0·005$), chi squared analysis.

GVHD = graft-versus-host disease

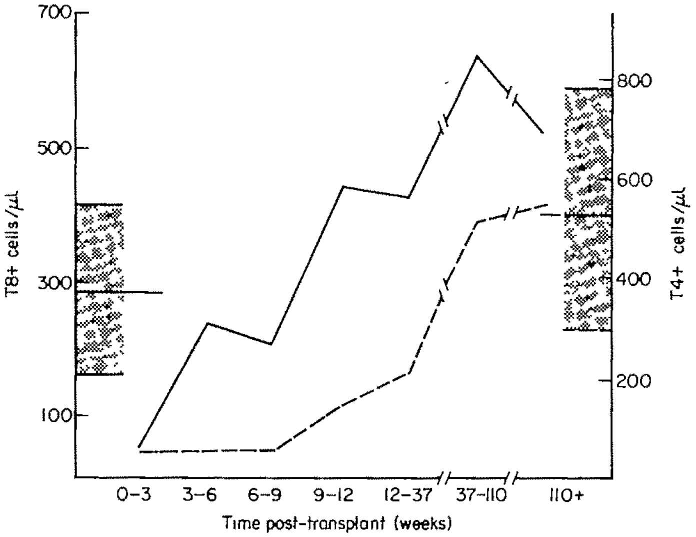

FIGURE 3. Absolute numbers of circulating T4-positive (– – –) and T8-positive (——) lymphocytes after marrow transplantation. Values represent mean values for patients grouped by time interval post-transplant. The normal range for each subpopulation is shown by the respective hatched areas.

### *Ia-like antigen staining*

All skin biopsies were also examined for the presence of cells bearing Ia-like antigens as defined by antibody Leu-HLA-DR. In the epidermis of normal persons Ia-like antigens were regularly detected on OKT6+ dendritic cells and OKT6+ non-dendritic large cells, both thought to be Langerhans cells. In the dermis, Ia-like antigen was regularly detected on OKT6-negative stellate and spindle cells, both thought to be tissue histiocytes. It was also regularly present on vascular endothelium and occasionally on OKT6+ large non-dendritic cells and OKT6+ dendritic cells (both thought to be Langerhans cells). Additionally, in patients, Ia-like antigen was occasionally detected on lymphoid cells and occasionally on epithelial cells. The staining on keratinocytes occurred in one of two patterns: in 5% of biopsies a linear staining of the epithelial cells of the dermo-epidermal junction was noted. More rarely, a faint intercellular staining pattern was seen on the upper layer of cells in the epidermis. There was no correlation of either pattern with acute or chronic GVHD.

### *Circulating T4+ and T8+ cells*

The absolute number of circulating T4+ cells was markedly reduced post-transplant and did not reach the normal range till late post-transplant (Fig. 3). In contrast, the number of T8+ cells reached and exceeded the normal range early post-transplant, and subsequently remained at an elevated level (Fig. 3). Both the low numbers of T4+ cells and the high numbers of T8 cells caused reversal of the normal (2:1) T4:T8 ratio.

## DISCUSSION

From murine experiments there appears little doubt that T cells are the trigger for the initiation of graft-versus-host disease after bone marrow transplantation. GVHD does not occur if T cells are removed from the donor inoculum prior to its infusion into the recipient (Korngold & Sprent, 1978; Onoe *et al.*, 1980; Vallera *et al.*, 1982). The pathogenesis of target organ damage in GVHD, however, is much less clearly defined. This study was performed in an attempt to define which mononuclear cell subpopulations, if any, were associated with GVHD of the skin in man. We found that neither the helper-inducer T cell subset (T4 +) nor the cytotoxic-suppressor T cell subsets (T8 +) were present in the epidermis in the majority of patients with acute GVHD early post-transplant, and thus we were unable to implicate either subpopulation in the causation of cell-to-cell contact damage at this stage of the disease. Likewise, B cells (B1 +), natural killer cells (Leu 7 +), and monocytes (OKM1 +) were also absent. In chronic GVHD, while the T4 + subset was present in lower numbers than normal, the T8 + subset was found with increased frequency, and our findings are consistent with this subpopulation contributing directly to the skin lesion of chronic GVHD.

Previous studies have emphasized the variable nature of the lymphoid infiltrate in the skin of patients with acute GVHD (Lerner *et al.*, 1974; Sale *et al.*, 1977; Lampert *et al.*, 1982). Lerner *et al.* (1974) described it as 'slight to moderate' and stated that in a few cases there was virtually no inflammatory reaction. Sale *et al.* (1977) stated it to be 'by no means a constant or marked feature'. Lampert *et al.*, (1982) found no infiltrate in two of nine cases. The paucity of lymphocytes in both these studies and our own raises the possibility of a soluble factor being responsible for the skin damage in this disease. Indeed, Tsoi *et al.* (1978) noted deposits of IgM and complement at the dermo-epidermal junction (the main site of morphologically-recognizable damage) in patients with both acute and chronic GVHD, and skin antibodies have been recorded in human GVHD (Saurat *et al.*, 1976). Lymphokines secreted by T cells and causing a systemic delayed type sensitivity (DTH) reaction as the mediator of organ damage in GVHD (Mason, 1981) would be another theoretical candidate, although a cellular infiltrate compatible with DTH has not been noted in organs involved by acute GVHD.

In favour of lymphocyte-mediated organ damage in GVHD are the ultrastructural features of point contact and broad zone contact described in the tongue and lip of rhesus monkeys undergoing acute GVHD (Woodruff, Butcher & Hellerstein, 1972), and in the rectal mucosa of humans with acute GVHD of the gut (Gallucci *et al.*, 1982). Gallucci *et al.*, (1979) have also described the same features in the skin of patients with chronic GVHD. There are no such reports at present on the skin of humans with acute GVHD. T cell projections towards epithelial cells (point contact) have been implicated as a mechanism of T cell killing (Sanderson & Glauert, 1979), and in the mouse, cytotoxic T cells that preferentially lyse epithelial cells and that are specific for epidermal alloantigens have been described (Tyler & Steinmuller, 1981). It is theoretically possible, although unlikely in view of the number of sections analysed, that we missed infiltrating lymphocytes in this study, or that patients receiving cyclosporin as immunosuppression to minimize GVHD (the majority in this study) have different lymphocytic reactions during GVHD from those receiving methotrexate (the majority in previous reports). Finally, it is possible that only very small numbers of T cells are necessary to cause damage in acute GVHD, and that one cytotoxic T cell moves from one target cell to another and thus can destroy multiple epithelial cells (Ware & Grainger, 1981). It is important to attempt to resolve this issue, and ultrastructural studies utilizing immunoperoxidase sections stained with the T4 and T8 monoclonal antibodies are currently in progress.

It should also be remembered, as shown in this study (Fig. 3), that the T4 : T8 ratio in the blood of human marrow transplant recipients is reversed, with low numbers of T4 + cells and increased numbers of T8 + cells present long-term post-transplant. This occurs regardless of the presence or absence of graft-versus-host disease (Atkinson *et al.*, 1982; Friedrich *et al.*, 1982). Thus, the reconstitution of T cell subsets seen in the skin post-transplant may merely be a reflection of that in the blood, although the increased number of T8+ cells present in the long term survivors with chronic GVHD compared to those without chronic GVHD argues against this.

There have been few other studies utilizing monoclonal antibodies to determine the phenotype of mononuclear cells infiltrating the skin in human GVHD (Lampert *et al.*, 1982; Muller *et al.*, 1982). Lampert *et al.* (1982), using indirect immunofluorescence rather than immunoperoxidase, described nine patients biopsied between 16 and 641 days post-transplant. In agreement with our study, they found no or only occasional T cells (defined by OKT3) in the majority (five of nine), no B cells (defined by surface membrane immunoglobulin staining) and T8+ cells to be commoner than T4+ cells in those in whom T cells were present. Muller *et al.* (1982) using immunoperoxidase, described three patients with acute GVHD, but did not specify the time post-transplant of the biopsies, nor use antibodies defining the helper-inducer and cytotoxic-suppressor T cell subset. Increased T cells, expressing HLA-D region products, were noted in the epidermis and at the dermo-epidermal junction.

The sequential changes seen in our study with increasing time post-transplant emphasize the importance of time in the interpretation of the skin biopsy. Both the T4+ and the T8+ subpopulations decreased in frequency early post-transplant and began to return towards normal from day 22 onwards. OKT6+ Langerhans cells showed a similar, although more dramatic, pattern. This presumably indicates the sensitivity of the cells (of recipient origin) to the chemo-radiation conditioning therapy given immediately pre-transplant, with a subsequent repopulation by cells of presumably donor origin (Volc-Platzer *et al.*, 1984). Dermal Langerhans cells were less frequent than normal in patients with chronic GVHD, although not in long-term survivors without chronic GVHD. This appears to represent yet another parameter of the immune deficiency in this disease (Noel *et al.*, 1978; Witherspoon *et al.*, 1981), and may explain the marked defect of delayed type hypersensitivity which occurs in such patients (Witherspoon *et al.*, 1984). Perrault *et al.* (1984), Lampert *et al.* (1982) and Muller *et al.* (1982) also reported decreased numbers of Langerhans cells post-transplant. Dermal OKM1+ histiocytes (of recipient origin) also appeared to be chemo-radiation sensitive. Reconstitution (by cells presumably of donor origin) did not result in normal levels being achieved during the period of follow-up of this study.

Mononuclear cells bearing the OKT10 activation antigen were noted in the epidermis of two patients with acute GVHD, a phenomenon not present in normal skin. Elevated numbers of cells bearing this antigen have been described in the blood after human marrow transplantation (de Bruin *et al.*, 1981; Atkinson *et al.*, 1983), and we have shown it to be present on T and non-T cells and on both the T4+ and the T8+ subset in this situation (Farrell *et al.*, 1985). Furthermore, its presence on the T cell subsets declines rapidly post transplant. Mononuclear cells in the skin bearing it during acute GVHD presumably reflect this. Mononuclear cells bearing the Leu 7 antigen were noted in the epidermis of long-term survivors, but not in the skin of short-term survivors or normal persons. Leu 7 is normally expressed on a small subset of the cytotoxic-suppressor T cell population (Lanier *et al.*, 1983), as well as on natural killer cells. In contrast to the presence of OKT10 on the helper-inducer and the cytotoxic-suppressor subsets early post-transplant, we have found Leu 7 to be present only on the cytotoxic-suppressor subset and with increasing frequency as time elapses post-transplant (Farrell *et al.*, 1985). The Leu 7+ cells noted in long-term survivors could thus be either natural killer cells or cytotoxic-suppressor T cells. The anti-Leu 7 antibody also consistently stained cutaneous dermal nerves and this represents another example of antigen sharing between human blood cells and nervous tissue (Oger *et al.*, 1982).

Staining for Ia-like antigens with anti-HLA-DR was disappointing and added little additional information. DR antigen positivity was noted on only 7% of all biopsies and consisted of either a distinct band-like deposit at the dermo-epidermal junction or a diffuse faint deposit either on or between the upper layers of epidermal keratinocytes. It was certainly not seen consistently in patients with GVHD even though dermal histiocytes were regularly stained. In this regard our findings are different to those in the skin and gut of rats with GVHD (Lampert *et al.*, 1981; Mason, Dallman & Barclay, 1981); one of five patients in Muller's study (1982) and four of nine in Lampert's study, exhibited Ia-like antigen expression by keratinocytes.

Finally, two methodological points should be stressed. Our experience indicated that not all monoclonal antibodies defining the same differentiation antigen were particularly suitable for this technique and it is important to evaluate each antibody intended for such a study carefully beforehand. Secondly, unexpected reactions can occur. The Leu 7 staining of peripheral nerves is such an example. More importantly, dermal histiocytes reacted weakly with the T4 antibody. Such reactions underline the need to be able to assess morphology accurately, and this is done more rapidly with the immunoperoxidase methodology than with indirect immunofluorescence.

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# A prospective study of the histological changes in the skin in patients receiving bone marrow transplants

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## SUMMARY

Fourteen patients who received a bone marrow transplant (BMT) as treatment for leukaemia were included in a prospective study of the histological changes in the skin. The aim of this study was to improve the early diagnosis of graft-versus-host disease (GVHD).

It was found that the clinically 'normal' pre-transplant skin was in some cases histologically abnormal on H & E examination in patients who were on regular maintenance cytotoxic chemotherapy. These changes were similar to some of the features of GVHD.

Immunocytochemistry, although not specific, was found to be helpful in the diagnosis of some cases of GVHD. Suggestive features included a reduction in the numbers of Langerhans cells, an increase in the number of suppressor (OKT8 +) cells in the dermal infiltrate and the presence of Ia positivity of the keratinocytes in the epidermis.

Bone marrow transplantation is being increasingly used as a definitive treatment for aplastic anaemia (Storb *et al.*, 1983), the leukaemias (Thomas *et al.*, 1977; Buckner *et al.*, 1982; Clift *et al.*, 1982; McGlave *et al.*, 1982; Goldman *et al.*, 1982; Leader, 1982), severe combined immune deficiencies of infancy (Bortin & Rimm, 1977), some inborn errors of metabolism (Hobbs, 1981; Hobbs *et al.*, 1981) and other congenital disorders (Sieff *et al.*, 1983; Fischer *et al.*, 1983). Major obstacles to the successful use of allogeneic bone marrow transplantation are primarily the immuno-biological complications of the procedure, particularly immunosuppression, pneumonitis and graft-versus-host disease (GVHD). Relapse of the leukaemia following transplant, although substantially less than with chemotherapy, remains a problem. The early results from

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the centre at Seattle showed a survival of 56% for GVHD grades 0–1 and 14% for grades 2–4 (Glucksberg *et al.*, 1974). Improved methods of prevention of GVHD, including cyclosporin A, monoclonal antibody T-cell depletion, a better 'sterile' environment, parenteral nutrition and good nursing care may help to improve the prognosis.

Florid GVHD is easily recognized but these patients already have a poor prognosis. Recognition and immediate treatment of early mild GVHD might improve the outcome. However, clinically, these patients are the most difficult to identify as many of the signs and symptoms are similar to those caused by viral and other infections, which are relatively common in immunosuppressed patients. Skin rashes can also be produced by drug reactions, and these patients are exposed to multiple drug therapy, in particular antibiotics. The leukaemic patients will have all received multiple courses of combination therapy as treatment of their underlying disease. In addition, an intensive immunosuppressive conditioning regime is necessary prior to marrow transplantation to prevent rejection.

It is well recognized that cytotoxic agents can affect the skin both clinically (Cohen *et al.*, 1973; Blum, Carter & Agre 1973) and histologically (Koss, 1967; Weinstein & Valasco, 1972; Baker *et al.*, 1973; Smith & Gelfant, 1974; Kennedy, Smith & Goltz, 1975). Some of these histological changes on conventional H & E microscopic examination can be indistinguishable from those of GVHD (Sale *et al.*, 1977). It was decided, therefore, to conduct a prospective study of patients undergoing bone marrow transplantation with the aims of determining whether sequential biopsies could aid the diagnosis of GVHD using conventional histological examination and also establishing whether or not immunocytochemistry could improve the diagnostic value of a skin biopsy.

## METHODS

### *Patients*

Eleven consecutive children undergoing allogeneic bone marrow transplantation in the Royal Hospital for Sick Children, Glasgow, between September 1982 and June 1984 were studied.

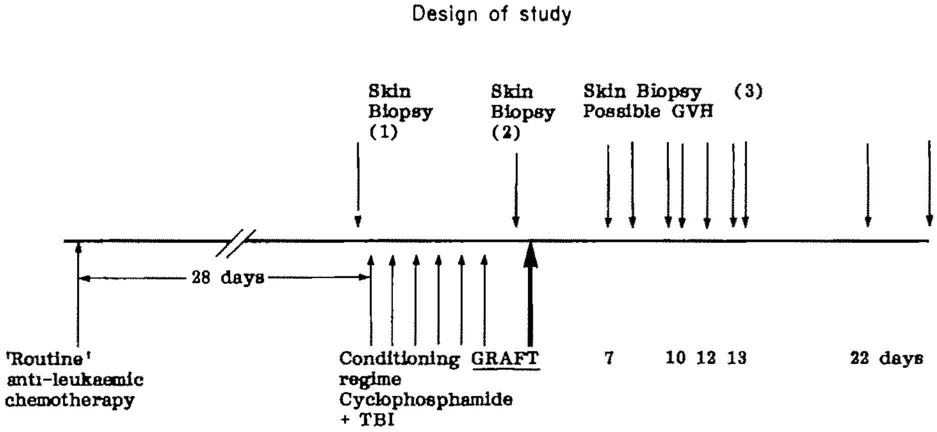

FIGURE 1. The design of the study. Three skin biopsies were performed, the first before the conditioning regime was commenced, the second immediately prior to the bone marrow transplant. Nine patients developed possible GVHD and were biopsied when clinically indicated. The earliest of these biopsies was performed on day 7 and the latest on day 27.

Four adult patients, two receiving allografts and two autologous grafts, in the Royal Infirmary, Glasgow, were also included in the study. The design of the study is shown in Fig. 1.

Twelve of the patients receiving an allogeneic marrow had leukaemia. Six of these had acute lymphoblastic leukaemia (ALL) in the second remission, two had poor prognosis ALL in the first remission, one acute myeloid leukaemia (AML) and three patients had chronic myeloid leukaemia (CML). The two autograft patients both had AML. One child with Gaucher's disease died within 48 hours of transplantation and was therefore excluded from the final analysis. All the remaining patients had leukaemia and were on regular maintenance cytotoxic therapy. Each had received a course of intensive chemotherapy approximately one month prior to the transplant. Patients were admitted for a full clinical assessment prior to receiving their pre-transplant conditioning regime. The skin was clinically normal on examination. During this time a 'baseline' biopsy of clinically normal skin was taken from the dorsal area (14 patients). Conditioning in the majority of cases consisted of high dose cyclophosphamide (60 mg/kg) followed by fractionated total body irradiation (12·0 Gy). A second skin biopsy was performed after the total body irradiation and prior to transplantation (13 patients).

### *Biopsies*

After the transplant was performed all patients were assessed regularly. If GVHD was suspected clinically, or if they developed skin lesions during this time, patients were seen by a dermatologist (RL) who examined the skin, including an examination of the mouth for possible oral involvement. If the skin lesions were thought to be those of GVHD, a third skin biopsy was taken. This was required in nine patients, and on six occasions a further biospy was performed: two from an earlier case who presented with chronic GVHD, one repeated because of continuing clinical suspicion of GVHD with a non-diagnostic third biopsy, two because of possible development of chronic GVHD and one from a patient with suspected systemic candidiasis.

The specimen from each skin biopsy was divided. One portion was placed in 10% neutral buffered formalin for paraffin embedding and conventional histological examination after H & E staining. The other was snap frozen for immunocytochemistry. All slides for conventional light microscopy were examined blind by one observer (RMacK). The specimens were scored for hyperkeratosis, loss of epidermal polarity, liquefaction degeneration, satellite cell necrosis, inflammatory infiltrate (0 = none, 1 = present, 2 = marked) and the presence of colloid bodies (0 = none, 1 = present). The maximum possible score, therefore, was 11 and normal skin scored 0.

The following monoclonal antibodies were used in a standard immunoperoxidase technique to identify lymphocyte subsets and Langerhans cells: OKT11 and OKT3 (pan T cell, Ortho), Leu 1 and Leu 4 (pan T cell, Becton Dickinson), Leu 3a and OKT4 and 4A (T helper cells, Becton Dickinson and Ortho), OKT8 (suppressor T cells, Ortho), Na1/34 and Ia 231 Langerhans cells and HLA-DR positive cells, kindly donated by Professor A.J.McMichael, University of Oxford and Dr V. Heyningen, MRC Cytogenetics Unit, Edinburgh, U.K. respectively. All slides were examined blind by two observers (RMacK and MT).

## RESULTS

Nine patients developed clinical features suggestive of GVHD. These included malaise, fever and a maculopapular rash, with involvement of the palms and soles, and oral involvement. Only two patients initially had abnormal liver enzymes, although three of the remaining six patients developed abnormal liver function at a later stage. One patient proceeded to develop florid

TABLE 1. Histology of skin biopsies from 14 bone marrow transplant patients

| Transplant type | Case no. | Sex | Leukaemia type | Histological score: Clinically normal skin (14 patients) | Histological score: Post conditioning (13 patients) | Histological score: Post transplant possible GVHD (9 patients) |
|---|---|---|---|---|---|---|
| Allogeneic | 1 | F | CML | 3 | 3 | 7 |
| Allogeneic | 2 | M | ALL | 4 | 4 | 5 |
| Allogeneic | 3 | M | ALL | 5 | 3 | 11 |
| Allogeneic | 4 | M | CML | 5 | 4 | 11 |
| Allogeneic | 5 | M | ALL | 0 | 3 | 11 |
| Allogeneic | 6 | M | ALL | 2·5 | 6 | 11 |
| Allogeneic | 7 | M | AML | 6 | ND | 7 |
| Allogeneic | 8 | M | ALL | 3 | 7 | 10 |
| Allogeneic | 9 | M | ALL | 9* | 6 | 6 |
| Allogeneic | 10 | M | ALL | 1 | 1 | NA |
| Allogeneic | 11 | M | CML | 2 | 6 | NA |
| Allogeneic | 12 | M | ALL | 3 | 2 | NA |
| Autologous | 13 | F | AML | 2 | 3 | NA |
| Autologous | 14 | F | AML | 3 | 4 | NA |
|  | Total score |  |  | 48·5 | 52 | 79 |
|  | Mean score per biopsy |  |  | 3·46 | 4·0 | 8·78 |

CML, chronic myeloid leukaemia, ALL, acute lymphoblastic leukaemia; AML acute myeloid leukaemia.
ND = Not done
NA = Not applicable—no clinical features suggestive of GVHD.
* This boy had a different and more intensive course of chemotherapy 4 weeks prior to his admission.

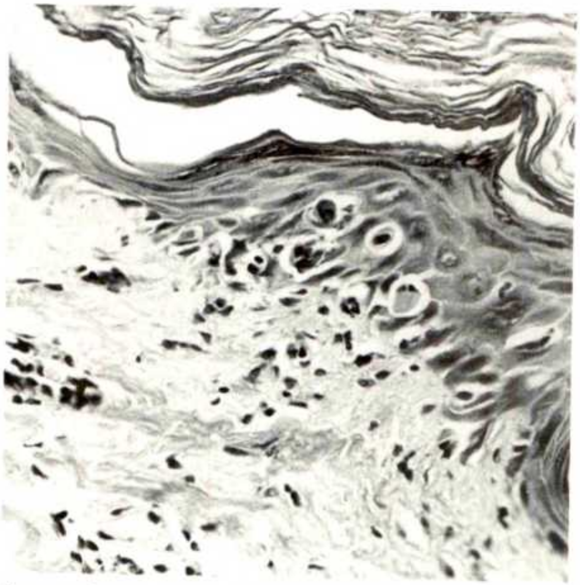

FIGURE 2. Acute graft-versus-host disease at day 12 post transplant. Haematoxylin and eosin stained specimen showing liquefaction degeneration and satellite cell necrosis.

GVHD; in the other eight patients the features continued to be non-specific with fever and a maculopapular rash. Involvement of the palms and soles was seen to a variable degree in six and in all eight patients respectively. Two patients with a maculopapular rash had been started on broad spectrum antibiotics, raising the possibility of a cutaneous adverse drug reaction.

The results of conventional microscopy (H & E) are presented in Table 1. The 13 pre-preparation biopsies from clinically normal skin gave a total histological score of 51 with a mean score per specimen of 4. Scores ranged from 0–9, although only one patient scored 9; the remaining 12 fell into the range 0–6. In general, a minor loss of epidermal polarity and liquefaction degeneration were seen. Colloid bodies were rare.

After the conditioning regimen, biopsies from 12 patients gave a total histological score of 49, again giving a mean score per specimen of 4. On this occasion one patient scored 1 and the remaining 11 lay within the range of 3–7. The nine biopsies from cases where GVHD was suspected clinically gave a mean score of 9 (total score 80) with a range from 5–11. An example of the histological changes is illustrated in Fig. 2

The results of the immunocytochemical staining are shown in Table 2. Langerhans cells were reduced after the conditioning regime but showed a further reduction to less than eight Langerhans cells/200 basal cells in seven of the nine patients in whom GVHD was suspected clinically. One patient had a low/normal level of 30 Langerhans cells/200 basal cells. The normal range for this laboratory is 39 ± 3 Langerhans cells/200 basal cells. The dermal infiltrate was sparse at all three stages with few cells staining with either the pan-T or T-helper cell antibodies. Cells staining with the suppressor lymphocyte antibody (OKT8) were also very sparse in both the pre-treatment biopsies and those taken after the conditioning regimen. However, in five out of nine patients in whom GVHD was suspected, a suppressor cell infiltrate was seen (Fig. 3a).

Ia staining of the keratinocytes was negative in the epidermis in both of the pre-transplant biopsies, but was positive in six of the nine biopsies from patients with suspected GVHD (Fig. 3b).

TABLE 2. Results of immunocytochemical staining of skin biopsies

| | No. of patients | Total T lymphocytes | Suppressor T lymphocytes | Helper T lymphocytes | Langerhans cells /200 basal cells | Epidermal Ia staining |
|---|---|---|---|---|---|---|
| Before preparation, clinically normal skin | 14 | Very few | Occasional | Very few | 29·2 ± 13·1* | Negative |
| After preparation | 13 | Very few | Occasional | Very few | 24·3 ± 13·5 | Negative |
| Post-transplant patients with rash | 9 | Very few | 5/9** clustered | Very few | 6·5 ± 9·2 | 5/9** positive |

*Mean ± SD.

** In five out of nine biopsies.

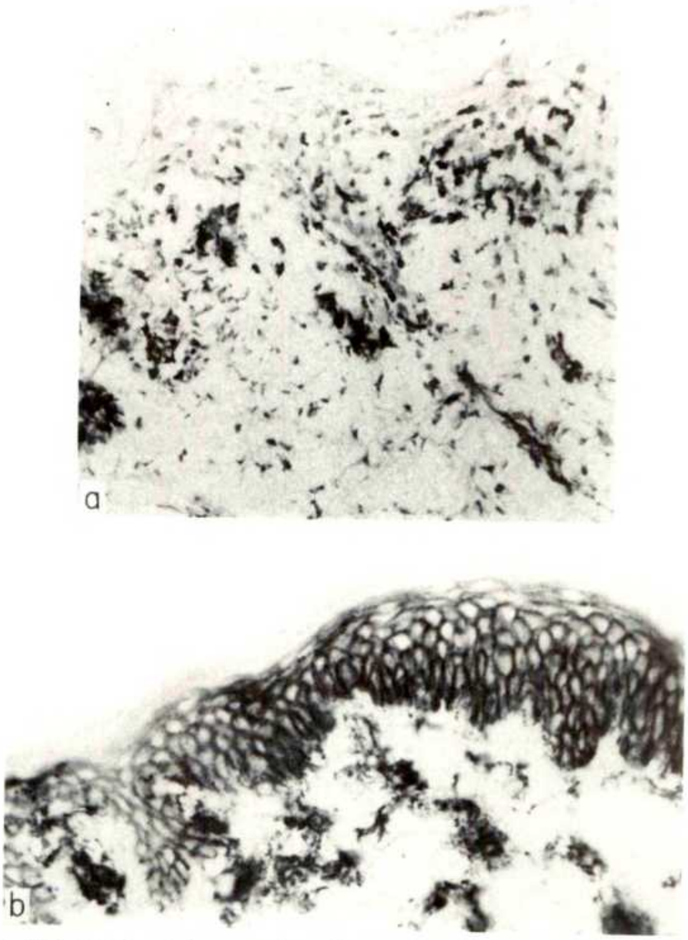

FIGURE 3.(a) Immunocytochemical preparation of biopsy from same patient as in Fig. 2 stained with OKT8 (suppressor cytotoxic) antibody. Clear positive staining of cells is seen; (b) Ia positive keratinocytes in the epidermis in suspected graft-versus-host disease at day 15. Immunocytochemical preparation stained with antibody Ia 231.

Of the four patients who had equivocal scores (5–7) on light microscopy, all four had grossly reduced numbers of Langerhans cells in the epidermis, all four had positive Ia keratinocyte staining and two also showed an OKT8 positive infiltrate. The one child who developed possible mild GVHD after pre-treatment of his marrow *in vitro* with OKT3 to remove the mature T-cells also developed positive Ia keratinocyte staining. However, the infiltrate in his case was of OKT4 positive helper cells. It is of interest to note that three of the four children who developed GVHD and who had only a sparse infiltrate of OKT8+ cells were long term survivors, the one death being due to leukaemic relapse. In contrast, four of the five patients with a more intense OKT8+ infiltrate died of GVHD.

Of the two patients who received an autologous transplant, only one was biopsied post transplantation. The biopsy was delayed until the thirty-fifth day post transplant because of pancytopenia. Therefore, the findings are not strictly comparable with those biopsies from the possible GVHD patients which were in general performed earlier (days 7–13 in 11 children and days 22 and 27 in two adults). However, in this patient Langerhans cells were normal at 42/200 basal cells; there was only a sparse dermal infiltrate and no Ia staining of the epidermal keratinocytes. In contrast, the two patients who developed GVHD after an allogeneic transplant and who were biopsied on days 22 and 27 respectively still showed a gross reduction in Langerhans cells at 1 and 4/200 basal cells respectively. Both showed an OKT8 positive infiltrate and Ia staining of the epidermis.

## DISCUSSION

Prompt diagnosis and early treatment of acute GVHD may alter the outcome of the disease. Both sequential biopsies and immunocytochemistry may be of diagnostic value. The clinical features in a well-developed case can be almost diagnostic: the sudden development of fever and malaise in a child who was well for the first 7–10 days after grafting, the development of facial erythema and a generalized maculopapular rash with involvement of the palms and soles. The gastro-intestinal tract and liver can also be affected with diarrhoea and abnormal liver function tests. Early mild cases are less easy to diagnose. The differential diagnosis includes both viral infections and drug reactions which share a number of the clinical features.

Clinically florid cases generally show a characteristic histological picture (Kruger *et al.*, 1971; Leader, 1977; Woodruff *et al.*, 1976; Rappeport *et al.*, 1979; Saurat, 1981; Wick *et al.*, 1983) although none of the features, including satellite cell necrosis are pathognomonic to the disease. Cytotoxic therapy can produce many of the features, although, in general, they are less marked. Nevertheless, cytotoxic therapy alone can produce a histological picture that is indistinguishable from acute GVHD (Sale *et al.*, 1977). Sequential biopsies can be helpful in establishing a baseline in an individual patient and this makes the interpretation of subsequent biopsies more meaningful.

Immunocytochemistry is useful in providing additional confirmatory evidence and the present work confirms the findings of Lampert *et al.* (1982) who also found a reduction in the Langerhans cells and an OKT8+ suppressor T-cell infiltrate. Several of their patients also showed positive Ia staining of the keratinocytes in the epidermis. Our study followed patients sequentially and has demonstrated that the reduction in Langerhans cells is likely to be due, at least in part, to the conditioning regimen. Sloane *et al.* (1984) found a reduction in the Langerhans cells in patients 8–30 days post transplantation. However, work on rat chimeras has suggested that a more profound reduction is seen in those animals with GVHD as compared with those who only received the conditioning regime (Suitters & Lampert, 1983). The OKT8 positive infiltrate appears to be a feature of GVHD. The circulating lymphocytes have been shown to be present in different proportions from those in the dermal infiltrate (Favrot *et al.*, 1983).

Of the nine cases with suspected GVHD, six are now dead. Four died of GVHD (three in the acute phase and one with chronic GVHD), one of a further marrow relapse and one with a candidal septicaemia. This patient failed to re-engraft after he had rejected his initial transplant which had been pre-treated with monoclonal antibodies (MBG6 and RFT8) to remove the mature T-cells. Candida was also cultured from a skin biopsy which was performed to elucidate the cause of the associated papular eruption.

Two of the three survivors had early acute GVHD with relatively minor histological changes (scores 5 and 6). They both responded to high dose steroids. The use of high dose steroids is not without risk, as one patient had a grand mal seizure and another developed reversible hypertension. Pulse steroids can be lifesaving in GVHD but should be used with discrimination. The diagnosis could be made on firmer grounds if the clinical suspicion can be supported by both histological and immunocytochemical findings. However, with pre-treatment of the marrow to remove the mature T lymphocytes, GVHD may become less of a threat to a successful outcome of bone marrow transplantation in patients with leukaemia.

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# Effects of pemphigus antibody on the organization of microtubules and keratin-intermediate filaments in cultured human keratinocytes

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## SUMMARY

It has been suggested that pemphigus antibodies (PA) react with the surface molecules on keratinocytes, and induce the production and release of proteases resulting in acantholysis. If this is the case, the immunoreactions on the cell surface may send signals to the interior of the cell across the membrane. The present study was carried out to determine whether or not cytoskeletons [microtubules (MT) and keratin-intermediate filaments (KIF)] respond to PA-immunoreactions in cultured human keratinocytes, by indirect immunofluorescence microscopy using anti-keratin and anti-α-tubulin antibodies.

During incubation for 30 min to 72 h in a PA-containing medium with a normal concentration of $Ca^{2+}$ (1·2 mM), no changes in MT or KIF organization were detected. Alterations in the organization of these filaments were observed 96 h after addition of PA. When cells grown in a normal medium for 5–7 days were transferred to a medium containing PA and a low level of $Ca^{2+}$ (0·07–0·14 mM) the reorganization of KIFs and MTs occurred after 1 h incubation. However, no reorganization of the cytoskeletons was detected in the absence of cell detachment. These observations suggest that the pemphigus antibody-induced reorganization of MTs and KIFs does not precede acantholysis and is probably secondary to it, but is not a direct transmembrane response. The present study also showed that immunofluorescence microscopy of KIFs may be one of the most sensitive methods for detecting early cell-to-cell dissociation in cultured keratinocytes.

It is now widely accepted that pemphigus is an autoimmune blistering disease of the skin (Beutner & Jordon, 1964, 1968; Jordon, 1979). In the serum samples of patients with pemphigus, IgG antibodies have been detected in the intercellular spaces of the epidermis by

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indirect immunofluorescence (Beutner & Jordon, 1964, 1968; Jordon, 1979). These antibodies are also detectable by direct immunofluorescence in biopsies from patients with pemphigus (Beutner & Jordon, 1968; Jordon, 1979; Jordon, Triftshauser & Schroeter, 1971). Immunoelectron microscopy has revealed that these antibodies bind to the cell surface of keratinocytes (Wolff & Schreiner, 1971; Takigawa, Imamura & Ofuji, 1978). Recently, Stanley *et al.* (1982) have demonstrated that most, but not all, pemphigus sera bind the cell surface antigen, which is a glycoprotein synthesized by human keratinocytes. In addition, these antibodies can cause acantholysis in organ cultures of skin, without complement (Shiltz & Mitchel, 1978), and pemphigus lesions in new born mice (Anhalt *et al.*, 1982).

From these observations, it is thought that the binding of pemphigus antibody to the cell surface of keratinocytes is the first step in induction of acantholysis. As one of the second steps, many experimental results have suggested that proteolytic enzymes, especially plasminogen activator, are released from the keratinocytes by binding of pemphigus antibody to the cell surface and this release of enzymes may be profoundly involved in the acantholytic processes (Farb, Dykes & Lazarus, 1978; Morioka, Naito & Ogawa, 1981; Hashimoto *et al.*, 1983). This suggests, in turn, that cell surface-directed antibodies may have some influence on the biological activities of keratinocytes. In that case, the immunoreaction occurring on the membrane surface may be transferred into the cytoplasm or nucleus as some form of transmembrane signals. The present study attempted to detect intracellular events following the pemphigus antibody-cell surface antigen reactions, by the observation of cytoskeleton-reorganization in cultured keratinocytes.

The cytoskeleton consists of three distinct filaments: microtubules, intermediate filaments and microfilaments, and is thought to be involved in many cellular functions including the maintenance of cell shape, cell motility, cell adhesion, cell division and organelle localization. Cytoskeletons have been shown to respond to the stimulation of the cell surface by hormones (Aubin, Alders & Heersche, 1983), viruses (Murti & Goorha, 1983), carcinogenic agents (Schliwa *et al.*, 1984; Mascardo & Scherline, 1984; Phaire-Washington, Silverstein & Wang, 1980) and epidermal growth factor (Keski-Oja, Lehtho & Virtanen, 1981), such that their distribution is reorganized. It is, especially, of interest to note that rather rapid alterations in the organization of actin (Schliwa *et al.*, 1984), microtubules (Mascardo & Scherline, 1984) and intermediate filaments (Phaire-Washington *et al.*, 1980) were induced in HeLa cells or macrophages by the tumour promoter 12-O-tetradecanoyl-phorbol-13-acetate, which is known to cause stimulation of plasminogen activator production (Wigler & Weinstein, 1976).

The present study was initiated to examine whether a similar transmembrane response of cytoskeletons can be induced by addition of pemphigus antibodies to human skin cultures.

## METHODS

### *Sera*

Sera from three patients with pemphigus vulgaris were used. The sera bound to cryostat-sectioned skin in a typical epidermal cell surface pattern at titres of 1:40 to 1:60, as shown by indirect immunofluorescence. IgG was isolated from the sera by using Affi-Gel Blue chromatography (BIO-RAD Laboratories, California, U.S.A.). Three normal sera were also used as controls. These sera were heated at 56°C for 30 min to inactivate complement.

### *Antibodies*

*Antikeratin.* Keratin was extracted from human thigh skin according to published procedures

(Steinert, 1975). The purified keratin was electrophoresed on SDS-polyacrylamide gels (PAGE) and the polypeptide with a molecular weight of 50,000 was extracted from the gel. This 50 kd polypeptide was used to produce antiserum to the keratin. Two milligrams of this keratin peptide was dissolved in 1 ml of 8M urea, 1 mM dithiothreitol, and 10 mM Tris–HCl (pH 9·0). This solution was then mixed with an equal volume of Freund's complete adjuvant and injected intradermally into multiple sites in Japanese white rabbits. The animals were given a second injection 3 weeks after the first. The development of anti-keratin type intermediate filament (KIF) antibodies was monitored weekly by Ouchterlony double diffusion analysis. IgG was purified by Affi-Gel Blue chromatography. The reason why this subunit was used is that the antibodies to other subunits did not react with cultured keratinocytes well enough to study cytoskeletal structures, although they showed strong reactivity to suprabasal cells in thin sections of epidermis (unpublished data).

*Antitubulin.* Monoclonal antibody (mouse IgG) against α-tubulin was purchased from Amersham International (England).

### *Secondary antibodies*

Fluorescein isothiocyanate (FITC)-or rhodamine-lissamide sulphonylchloride-labelled goat IgG, anti-mouse IgG, FITC-labelled anti-goat IgG anti-rabbit IgG and FITC-labelled goat anti-human IgG were purchased from Cappel Laboratories (West Chester PA, U.S.A.).

### *Cell culture*

Explant cultures were used in the present experiments. Several small pieces of human skin were placed on coverslips. The cultures were fed with Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, 0·4 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 84 ng/ml cholera toxin, 100 μg/ml streptomycin and 100 unit/ml penicillin. The medium was changed at 2-day intervals. Cultures were grown in a humidified atmosphere under 5% $CO_2$. On the experimental days, after 7–10 day culture periods, the cells were observed under a phase contrast microscope and also by time-lapse cinematography.

On the experimental days, the medium was replaced with a fresh medium consisting of Dulbecco's modified Eagle's medium supplemented with 0·4 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 84 ng/ml cholera toxin, 100 μg/ml streptomycin, 100 unit/ml penicillin and 10% heat-treated (56°C, 30 min) pemphigus or normal serum, or purified IgG fraction from pemphigus or normal serum at concentrations of 3–7 mg/ml.

In another experimental series, the cells were cultured at first in a medium with a normal $Ca^{2+}$ concentration for 7–10 days, then the cells were incubated in a culture medium containing pemphigus serum at a low concentration of $Ca^{2+}$ (0·07–0·14 mM). The calcium concentration in the final complete medium was determined as follows. The calcium concentration in the sera from patients and control individuals were known from the clinical laboratory data, and the absolute amount of calcium in these sera, which were added to a $Ca^{2+}$-free medium, was calculated. Then $CaCl_2$ solution was added to give the final calcium concentration of 0·07–0·14 mM. Other conditions were the same as in experiments performed at normal $Ca^{2+}$ concentration (1·2 mM).

The cells grown on the coverslips were fixed 30 min, 1 h, 2 h, 4 h, 12 h, 24 h, 48 h, 72 h and 96 h after replacement of the medium.

### *Immunofluorescence microscopy*

The cells, on the coverslips, were rinsed with PBS at room temperature and dipped for 7 min in −20 °C methanol. The fixed cells, on the coverslips, were soaked in a solution of 0·5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM N-tocyl-L-phenyl-alanine chloromethyl ketone (TPCK) for 10 min at room temperature.

The cells were incubated with the first antibody for 45 min at room temperature, followed by several washes in PBS. In some specimens, double antibody labelling was carried out as follows: first the specimens were incubated with the rabbit anti-keratin antibody for 45 min followed by extensive washing, then incubated with the monoclonal antibody against α-tubulin for 45 min. The cells were then incubated with the specific second antibody, rinsed twice in PBS, air-dried and mounted in glycerol.

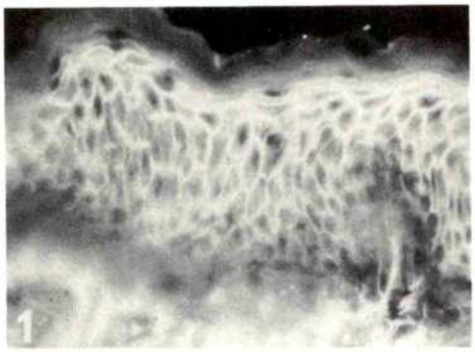
For the detection of the binding of pemphigus IgG on the cell surface of the cultured cells, the cells, on the coverslips, were fixed for 2 min with 20 °C acetone and stained with FITC-labelled goat anti-human IgG for 1 h at room temperature, followed by extensive washing. The culture medium containing IgG pemphigus antibody was tested to determine whether the antibody actively bound to the cell surface after 48-h incubation, by indirect immunofluorescence using normal cryostat sectioned skin.

Controls for specificity of the antibodies were routinely carried out. Photomicrographs were taken with an Olympus photomicroscope BHZ-ORFL (Olympus, Tokyo, Japan).

## RESULTS

The binding activity of pemphigus antibodies to the cell surface was retained even after 48-h incubation (Fig. 1). This observation indicates that the pemphigus-antibody activity had been retained throughout the experiment, since the medium was renewed every 48 h. As early as 30 min after the addition of pemphigus antibodies to the normal Ca$^{2+}$ medium, the binding of pemphigus antibodies to the cell surface of cultured keratinocytes was confirmed (data not shown). Binding was also shown in a low Ca$^{2+}$ medium containing pemphigus sera after incubation for 30 min, 1 and 2 h as shown in Fig. 2. These results indicate that the antibodies had bound to the cell surface even though the experiments were performed in a low Ca$^{2+}$ medium.

Keratin-intermediate filaments (KIFs) were identified by indirect immunofluorescence



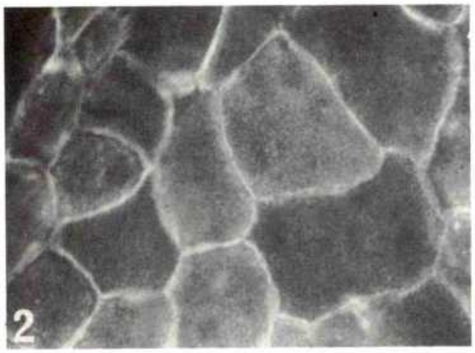


FIGURE 1. Binding of pemphigus antibody in 48-h-old medium to normal epidermis, as shown by indirect immunofluorescence. The antibody activity had been retained during incubation.

FIGURE 2. Binding of pemphigus antibody to the cell surface of cultured human keratinocytes, as shown by indirect immunofluorescence, 1 h after changing the cells from a normal medium to a low Ca$^{2+}$ medium containing pemphigus sera.

microscopy with anti-IgG to $\alpha$-keratin isolated from human epidermis. This anti-keratin antibody bound all subunits of $\alpha$-keratin obtained from human epidermis by immunoblotting (data not shown) and reacted with KIF bundles in cultured human keratinocytes. In the peripheral zone of the colony of cells, KIFs were observed radiating outwards from a dense perinuclear fibrillar network that was located at the side of the cell facing towards the centre of

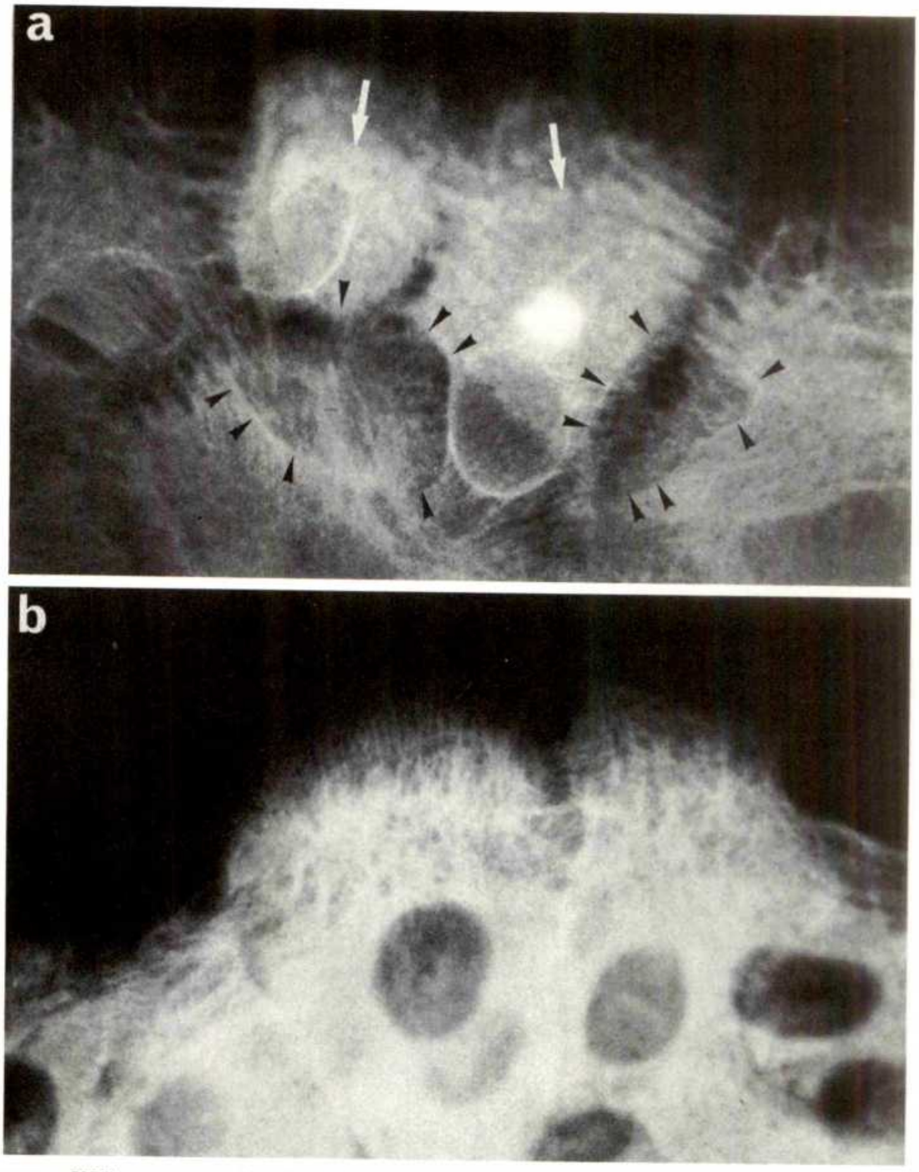


FIGURE 3. KIF arrangement in cultured keratinocytes incubated with pemphigus sera (a) or normal sera (b) for 96 h as shown by indirect immunofluorescence using antikeratin. KIFs are arranged in a radiating pattern from a juxtanuclear fibrillar aggregation (b). Pemphigus antibodies induced a retraction of KIFs from the cell periphery to the centre (a) and the intercellular spaces were widened (arrow-heads, a). Arrows show concentrated KIFs.

the colony. The laterally spreading bundles of KIFs were connected with those of the adjacent cells. Almost identical observations were made up to 72 h after the addition of pemphigus antibodies. However, in the cells treated with the antibodies for 96 h, reorganization of KIFs was apparent in the outer-most cells of the colony (Fig. 3a), but not in cells treated with control sera (Fig. 3b). The bundles of KIFs were retracted from the peripheral connecting zone to the centre of the cells, so that the intercellular spaces were widened (arrow-heads, Fig. 3a). This retraction of KIFs was more prominent in the inner side of the cell as indicated in Fig. 3a.

The organization of microtubules (MTs) was studied by indirect immunofluorescence with an anti-$\alpha$-tubulin monoclonal antibody. MTs seemed to be radiating from one spot which was located at the periphery of the cell facing the centre of the colony. No significant reorganization was found until 96 h incubation.

The treatment of the cultures with pemphigus antibodies in a reaction medium with a low $Ca^{2+}$ concentration (0·07–0·14 mM) induced a remarkable increase in susceptibility of keratinocytes to these antibodies. One-hour incubation with pemphigus antibodies at a low $Ca^{2+}$ concentration produced an extensive change in KIF organization similar to that seen at a normal $Ca^{2+}$ concentration after 96 h. KIFs were intensively retracted around the nucleus from the side facing the centre of the colony, in association with widening of the intercellular spaces (Fig. 4a). In control experiments, 1 h incubation in a low $Ca^{2+}$ medium without serum or with normal serum reduced the number of KIF bundles which were bound to those of the adjacent cells. However, a substantial number of them remained and no significant retraction of KIFs to the nucleus was seen (Fig. 4b). MTs also showed similar changes in organization accompanying the detachment of cell contacts after 1 h incubation with pemphigus antibodies, (Fig. 5a and b), but not with normal sera (Fig. 5c). Reorganization of the cytoskeleton was always associated detachment of neighbouring cells.

## DISCUSSION

Our results showed that the arrangements of MTs and KIFs were altered and the intercellular spaces were widened after 96 h incubation in medium with pemphigus antibodies at a normal $Ca^{2+}$ concentration. However, these rearrangements occurred so late that we could not detect

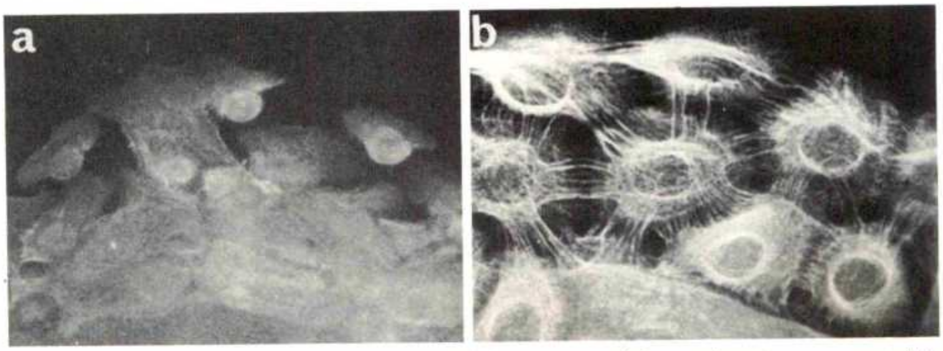


FIGURE 4. Effects of pemphigus antibodies on the KIF arrangement in human keratinocytes, reacted for 1 h with the antibodies at a low level of $Ca^{2+}$. (a) Pemphigus antibodies induced KIF retraction, especially at the side facing the centre of the colony. (b) One-hour incubation in a low $Ca^{2+}$ medium without serum, after transfer from normal medium reduced the number of KIF bundles bound to those of the adjacent cells, but no retraction was seen.

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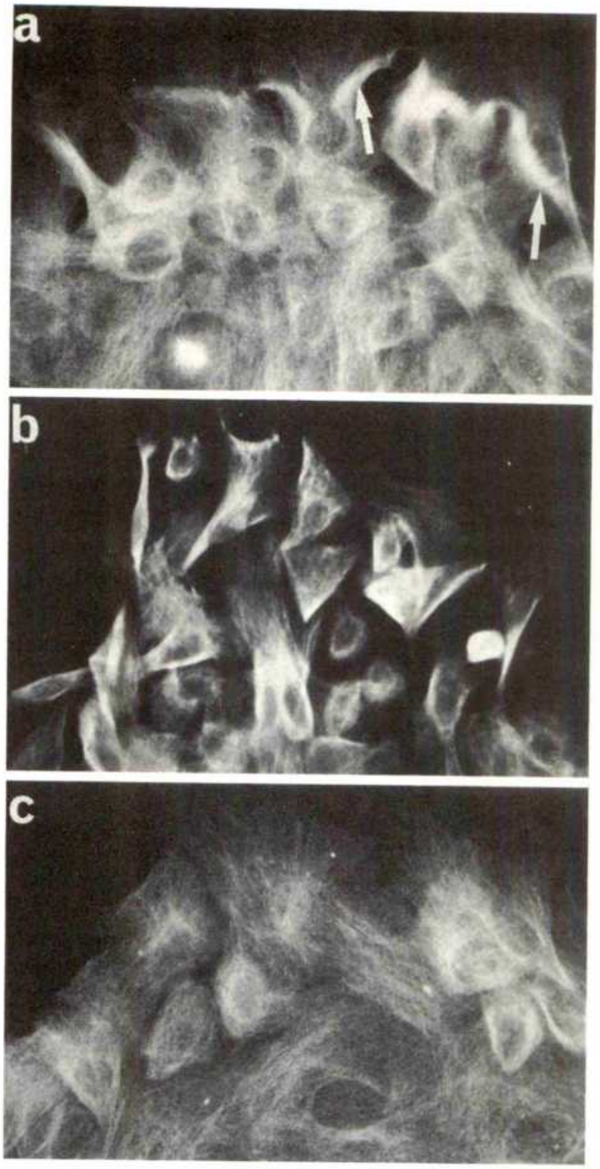

FIGURE 5. Effects of pemphigus antibodies on MT organization in human keratinocytes incubated for 1 h in a low $Ca^{2+}$ medium, as shown by indirect immunofluorescence using a monoclonal antibody against $\alpha$-tubulin. Pemphigus sera induced an obvious retraction (arrows) of MTs into the centre of the cell (a). Some portions of the colony were more intensely affected (b). Control sera did not affect the organization of MT (c).

whether filament reorganization or cell detachment occurred first. Therefore, the immunoreaction was performed at a low level of $Ca^{2+}$ to make the cell-to-cell adherence more fragile. At a low $Ca^{2+}$ concentration alterations in the arrangement of MTs and KIFs were observed after 1 h incubation. These alterations did not appear to precede the loss of cell-to-cell adhesion between keratinocytes, since no alterations in the arrangement of KIFs and MTs were observed without cell detachment. In addition, the patterns of reorganization of KIFs and MTs in the present study appeared to differ substantially from those induced by other membrane-affecting agents such as hormones (Aubin *et al.*, 1983), viruses (Murti & Goorha, 1983), tumour promoters (Schliwa *et al.*, 1984; Mascardo & Scherline, 1984; Phaire-Washington *et al.*, 1980) and epidermal growth factors (Keski-OIA *et al.*, 1981), which have been shown to cause dramatic transmembrane-effects on cytoskeletons. This suggests that the cytoskeletal changes are not directly due to transmembrane signalling by the cell-surface immunoreactions of pemphigus antibodies, but are possibly secondary to loss of cell-to-cell adhesion.

The reduction of the $Ca^{2+}$ concentration from 1·2 to 0·07 mM led to the antibodies causing a rapid retraction of MTs and KIFs from the cell periphery. Since it has been shown that a significant increase in plasminogen activator is detected in the cell 6–8 h after adding pemphigus antibodies to the medium (Hashimoto *et al.*, 1983), this reorganization of MTs and KIFs may be too early to be caused by newly induced proteases. Jones *et al.* (1984) have recently reported that antiserum from patients with pemphigus induced the separation of cell-to-cell contact in cultured mouse epidermal cells within 20 min, when the cells used had been switched to a medium containing normal levels of $Ca^{2+}$ after growth for a long period in a medium containing low levels of $Ca^{2+}$. They also have shown by indirect immunofluorescence that pemphigus antiserum and a rabbit antiserum directed against components of desmosomes, produced similar punctuate cell-surface patterns within the epidermis. They have proposed that the blistering in pemphigus is induced, at least in part, by specific autoantibodies that induce disruption of desmosomes in the epidermis. Our present study indicates that pemphigus antibodies can induce a rapid separation of cell-to-cell contact in cultured keratinocytes, when cell contacts were labile, and that immunofluorescence microscopy of KIFs is a sensitive method for examining the disruption of cell-to-cell contact.

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# The isolation of human sebaceous glands and apocrine sweat glands by shearing

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## SUMMARY

A new method of isolating human sebaceous and apocrine sweat glands by the repeated dissection of skin biopsies with scissors is described. The success of the technique is attributed to a line of weakness between the investing capsule and the surrounding connective tissue which parts under shear forces. The glands are judged to be viable by: (i) light and electron microscopy; (ii) ATP, ADP and AMP contents of 148·8 ± 30·3, 30·6 ± 4·7 and 14·9 ± 4·7 pmol (mean ± s.e.m.) for sebaceous glands and 310·2 ± 34·1, 90·35 ± 16·3 and 40·1 ± 11·8 pmol (mean ± s.e.m.) for apocrine sweat glands, which gave energy charges of 0·84 and 0·81, respectively; and (iii) a rate of sebaceous gland lipogenesis of 39·7 ± 3·7 pmol glucose incorporated into lipid/gland/h (mean ± s.e.m.).

Research into the physiology of human sebaceous glands in both normal and diseased states, notably acne, has been hindered by the lack of a method for their viable isolation (Shalita *et al.*, 1979). Kellum (1966) has described a method for the isolation of human sebaceous glands but this involves non-physiological exposure to 1 M $CaCl_2$ for 2 h. Studies on human sebaceous glands have therefore been carried out on whole skin plugs which have either been incubated *in vitro* (Hsia *et al.*, 1970; Cooper, McGrath & Shuster, 1976) or grafted onto nude mice (Petersen, Zone & Krueger, 1984). The disadvantages of these preparations are obvious.

The isolation of human apocrine sweat glands would also be of value in the study of their physiology in both normal and diseased states. These latter include hidradenitis suppurativa. Human apocrine sweat glands have been isolated by microdissection, but this is a technically demanding procedure with low yield. As a consequence, there has been only one *in vitro* study (Sato, 1980). Human eccrine sweat gland research had been similarly retarded by the difficulties of microdissection (Sato, 1977; Quinton, 1983) until the development of proteolytic digestion procedures which permitted the isolation of viable glands in high yield (Kealey, 1983; Okada, Kitano & Morimoto, 1983). A modification of these procedures has recently been described which avoids the exposure of glands to digestive enzymes (Lee, Jones & Kealey, 1984).

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We now report the application of this technique to the isolation of human sebaceous and apocrine sweat glands.

## METHODS

### *Materials*

Luciferin luciferase, purchased as 'ATP Monitoring Reagent', and ATP were from LKB, 232 Addington Rd., S. Croydon, CR2 8YD, U.K.; pyruvate kinase (type II) and adenylate kinase (grade V) were from Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, U.K.; phosphoenolpyruvate, ADP and AMP were from BCL, Lewes, East Sussex, U.K.; [U-$^{14}$C] glucose was from Amersham International, White Lion Road, Amersham, Bucks, HP7 9LL, U.K.; glutaraldehyde and embedding compounds were from Agar Aids, 66A Cambridge Road, Stanstead, Essex, U.K.; and osmium tetroxide from Johnson Mathey Chemicals Ltd., Orchard Road, Royston, Herts, U.K. All other chemicals were from BDH, Enfield, Middlesex, U.K., and of the highest purity available.

### *Preparation of glands*

Thin slices of apparently normal skin, 0·5 cm wide, were obtained from along the length of incisions of elective abdominal and chest operations on otherwise healthy adults receiving surgery at the Royal Victoria Infirmary and Freeman Hospital, Newcastle upon Tyne. Ethical Committee permission was obtained. The slices were placed in isolation buffer ($Na^+$, 144 mM; $K^+$, 5·9 mM; $Mg^{2+}$, 1·2 mM; $Ca^{2+}$, 1·25 mM; $Cl^-$ 125 mM; $SO_4^{2-}$, 1·2 mM; $PO_4^{2-}$, 1·2 mM; 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid [HEPES], 25 mM; glucose, 10 mM; adjusted to pH 7·4 with NaOH at room temperature).

The skin was repeatedly dissected with sharp scissors and the dissection placed in a plastic Petri dish under an Olympus binocular dissecting microscope. Glands were picked out immediately using two pairs of stainless steel Dumont no. 5 microforceps.

### *Light microscopy*

Glands were fixed in 4% formaldehyde, dehydrated and embedded in paraffin wax. Sections were stained with haematoxylin and eosin.

### *Electron microscopy*

Glands were fixed in phosphate-buffered 3% gluteraldehyde (pH 7·3), post-fixed in isotonic 1% osmium tetroxide and embedded in Araldite. Ultrasections (prepared on an LKB Ultratome III) were double stained with uranyl acetate and lead citrate and examined in an AEI Corinth 275 electron microscope.

### *Adenine nucleotide ratios*

These were measured by an adaptation of the method of Spielman, Jacob-Muller & Schulz (1981) based on the luciferin luciferase assay of ATP (Stanley & Williams, 1969). Glands were placed into 10 μl of 4% $HClO_4$ and then neutralized with KOH. The sample was adjusted to 80 μl and centrifuged (10,000 ***g***, 5 min). For the determination of ATP, 100-μl aliquots of supernatant were removed and added to an equal volume of reaction buffer (0·3 mM phosphoenol pyruvate, 9 mM $MgCl_2$, 5 mM KCl, 0·1 M Tris–EDTA, pH 7·75) at room temperature. For the determination of ATP + ADP the reaction buffer contained 20 units/ml pyruvate kinase, and for ATP + ADP + AMP, 20 units/ml of pyruvate kinase and 150 units/ml adenylate kinase. After incubation for 30 min at 25°C, 400 μl of 0·1 M Tris–EDTA pH 7·75 was added and the samples assayed for ATP content (Stanley & Williams, 1969). Control experiments showed that the conversion of ADP and AMP to ATP was complete under these conditions and that standard curves were linear over the range 0–100 pmol ATP. All assays were done in duplicate.

*Sebaceous gland lipogenesis*

The measurement of lipogenesis was made as described by Cooper *et al.* (1976) using 9 or 15 glands from each of four male subjects ranging in age from 33–66 years. After isolation, the glands were dissected free of collagen bundles and transferred to 0·5 ml bicarbonate buffered medium (Krebs & Henseleit, 1932) containing 2 mM [U-$^{14}$C] glucose (5 mCi/mmol) and incubated for 1, 2 or 3 h at 37°C under 95% $O_2$: 5% $CO_2$. After 5% $CO_2$ incubation the glands were removed and blotted. Total lipids were extracted by the method of Bligh & Dyer (1959) and aliquots removed in duplicate for scintillation counting. At each time point at least three glands were studied from each subject and the glands were either incubated singly or in groups of three or five.

## RESULTS

*Isolation*

Sebaceous, apocrine and eccrine sweat glands were released from the skin by the repeated action of the scissors. They were seen lying free in the suspension and were easily distinguished from each other. The sebaceous glands appeared as large lobulated spheres, often attached to hair follicles. Apocrine and eccrine sweat glands are much smaller than sebaceous glands and were seen as coiled tubes. Apocrine sweat glands are approximately twice the size of eccrine sweat glands and appeared pale yellow in colour whereas eccrine glands were white.

Sebaceous glands isolated from subjects of all ages were generally obtained with collagen bundles attached to them. However, their very large size rendered the removal of the collagen bundles with microforceps a very easy and rapid task. Apocrine and eccrine sweat glands, when obtained from subjects aged less than 30 years, were usually obtained free of attached collagen bundles or adipocytes and so their study was restricted to that age group.

*Yield*

*Sebaceous glands.* This varied according to the site of operation, chest skin being more richly endowed than abdominal. A chest incision of 20 × 0·5 cm (which is common in heart operations) routinely yielded up to 20 or 30 sebaceous glands.

*Apocrine sweat glands.* The distribution of such glands is restricted to the axillary, perianal and pudendal regions. The only routine surgical incisions from which we have obtained apocrine sweat glands have been inguinal. An average of four glands were obtained from each of four incisions of 5 × 0·5 cm. We have not obtained axillary skin from operations for axillary hyperhydrosis which should be a prolific source.

*Eccrine sweat glands.* The majority of glands that were seen lying free in the suspensions from all incisions were eccrine sweat glands and the yield from a chest incision of 20 × 0·5 cm was up to 80.

*Light microscopy*

*Sebaceous glands.* Figure 1 shows part of an isolated pilo-sebaceous unit. The acinar cells can be seen in their various states of differentiation. At the periphery of the acinus lie the small, flat undifferentiated cells (UDC). Towards the centre of the acinus are the partially differentiated cells (PDC) which are larger and contain numbers of lipid droplets. In the centre of the acinus the mature sebaceous cells (SC) are almost completely filled with lipid droplets and at this stage the nucleus may be no longer evident. Fibroblasts and collagen bundles can be seen surrounding the sebaceous gland. Figure 1 shows that the isolated glands retain their *in situ* structure (Ellis, 1967).

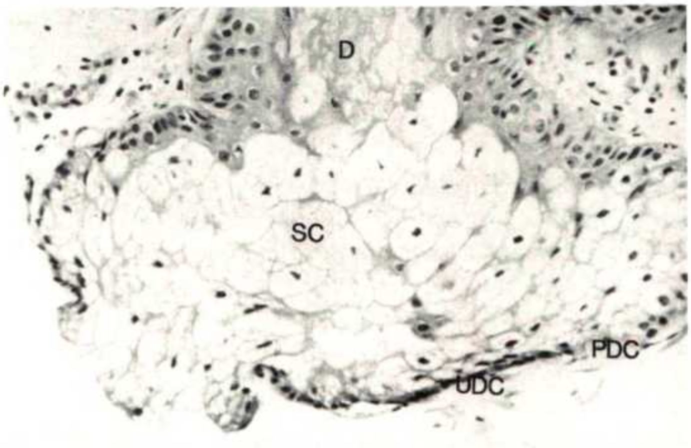

FIGURE 1. An acinus of a sebaceous gland from a pilosebaceous unit. The majority of the gland consists of mature sebaceous cells (SC) but peripherally there are partially differentiated cells (PDC) and undifferentiated cells (UDC). In the duct (D), which is lined with cells continuous with the external root sheath of the hair follicle, is the sebum and detritus of the sebaceous cells that have degenerated during holocrine secretion. Peripheral to the acinus are the fibroblasts and collagen bundles of the hair follicle connective tissue sheath. (H&E, magnification × 400.)

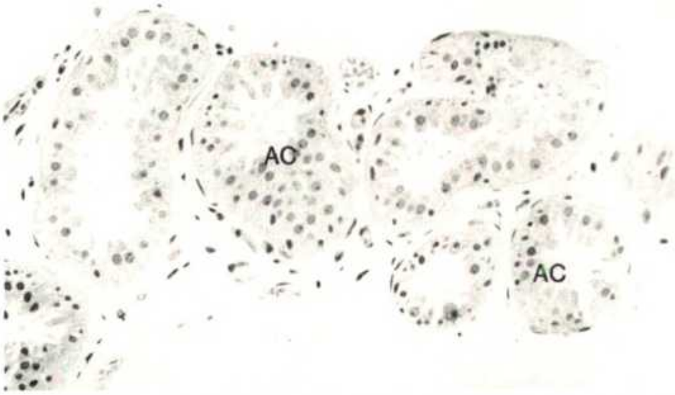

FIGURE 2. A transverse section of an isolated human apocrine sweat gland. The apocrine gland consists of a coiled tube within which can be seen the secretory cells with their characteristic apical caps (AC). Surrounding the coils are collagen bundles and fibroblasts. (H&E, magnification × 600.)

*Apocrine sweat glands*. Figure 2 shows part of an isolated apocrine sweat gland. The two cell types can be clearly seen. The secretory cells, which characteristically possess cytoplasmic protrusions known as apical caps (AC), are bounded peripherally by myoepithelial cells. The tubules are surrounded by a thin layer of collagen bundles and fibroblasts. The isolated glands retain their *in situ* structure (Ellis, 1967).

### *Electron microscopy*

*Sebaceous glands*. Figure 3 shows the surrounding layer of collagen fibres and fibroblasts, the basal lamina and apparently healthy undifferentiated, partially differentiated and mature

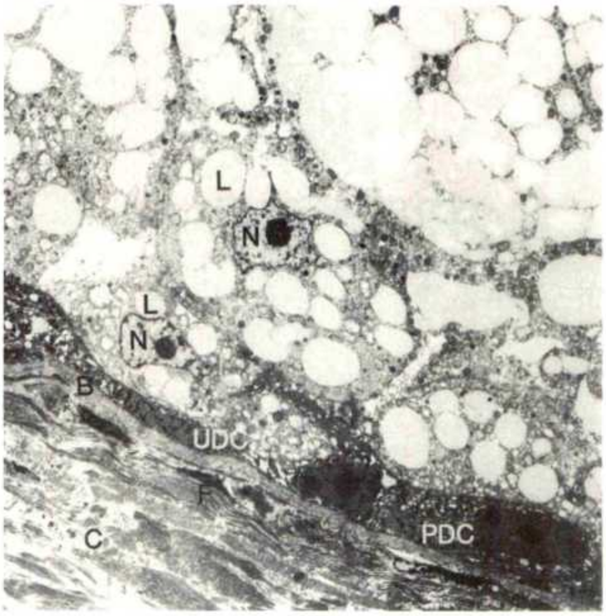

FIGURE 3. A transverse section of a human sebaceous gland. Resting on the basal lamina (B) is an undifferentiated cell (UDC) and partially differentiated cells (PDC). The nuclei (N) of the mature acinar cells are characteristically distorted by the growing lipid droplets (L) which fuse in the very mature cells that are about to undergo holocrine secretion. Outside the basal lamina are fibroblasts (F) and collagen fibres (C). (Magnification × 2000).

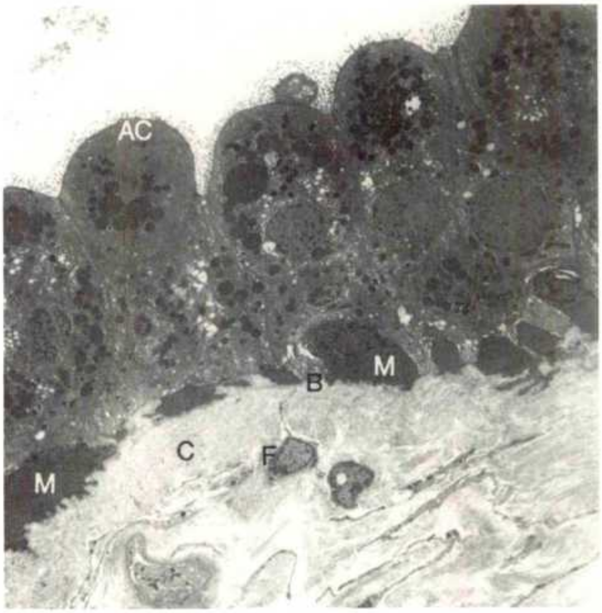

FIGURE 4. Transverse section of a human apocrine sweat gland. There are two cell types in the apocrine sweat gland. (i) Myoepithelial cells (M) which abut peripherally on the basal lamina (B) and (ii) secretory cells which are characterized by prominent nuclei, dark cytosolic granules and microvilli on the apical caps (AC). Outside the basal lamina are fibroblasts and collagen fibres. (Magnification × 2000.)

TABLE 1. Aspects of the biochemistry of human sebaceous and apocrine sweat glands

| Substance or activity measured | Sebaceous glands (mean ± s.e.m.) | Apocrine sweat glands (mean ± s.e.m.) |
|---|---|---|
| ATP (pmol/gland) | 148·8 ± 30·3 | 310·2 ± 34·1 |
| ADP (pmol/gland) | 30·6 ± 4·7 | 90·4 ± 16·3 |
| AMP (pmol/gland) | 14·9 ± 4·7 | 40·1 ± 11·8 |
| Energy charge | 0·84 | 0·81 |
| [U-$^{14}$C] glucose uptake at 1 h (pmol/gland) | 42·9 ± 10·0 | |
| [U-$^{14}$C] glucose uptake at 2 h (pmol/gland) | 75·9 ± 7·9 | |
| [U-$^{14}$C] glucose uptake at 3 h (pmol/gland) | 115·3 ± 17·9 | |

Glands were prepared as described in the text. For adenine nucleotide assays five sebaceous glands were obtained from each of three subjects ($n = 15$) and seven, four and two apocrine sweat glands were obtained from each of three subjects ($n = 13$). For studies on lipogenesis five or three sebaceous glands were incubated at each time point, having been obtained from four subjects ($n = 42$). Adenine nucleotide contents and rates of lipogenesis were determined as described in the text. The energy charge was calculated from [ATP] + 0·5 [ADP]/[ATP] + [ADP] + [AMP] as defined by Atkinson (1968).

sebaceous cells. The cytoplasmic lipid droplets, well developed Golgi complexes and many mitochondria are clearly evident within the acinar cells. These appearances are similar to those seen *in situ* (Ellis, 1967).

*Apocrine sweat glands.* Figure 4 shows the surrounding collagen fibres and fibroblasts, the basal lamina, and apparently healthy myoepithelial and secretory cells. The latter possess prominent nuclei, dark cytosolic granules and microvilli on the apical caps. These appearances are similar to those seen *in situ* (Ellis, 1967).

No evidence of compression damage has been seen in electronmicrographs of isolated sebaceous or apocrine sweat glands.

*Adenine nucleotides.* The ATP:ADP ratios of freshly isolated sebaceous and apocrine sweat glands were 4·9 and 3·4, respectively, and the ATP:AMP ratios were 10 and 77 respectively (Table 1). The energy charges for sebaceous and apocrine sweat glands, respectively, were 0·84 and 0·81 as calculated by [ATP] + 0·5 [ADP]/[ATP] + [ADP] + [AMP] (Atkinson, 1968) which are compatible with a healthy tissue (Spielman *et al.*, 1981).

### *Sebaceous lipogenesis*

The incorporation of $^{14}$C into sebaceous lipids was found to be linear over 3 h (Table 1) at 39·7 ± 3·7 pmol glucose/gland/h (mean ± s.e.m.; 42 glands from four subjects). In one subject 12 glands were incubated singly for 2 h and the relationship between the rate of [U-$^{14}$C] glucose incorporation and weight examined. Figure 5 shows that this was linear with a correlation coefficient of 0·87.

## DISCUSSION

The success of this technique of sebaceous and apocrine sweat gland isolation is attributable to a potential line of weakness between the periglandular capsule and the surrounding collagen which parts when sheared, leaving the underlying glandular cells apparently undamaged. This is also considered to be the means by which eccrine sweat glands are similarly isolated (Lee *et al.*, 1984). Hair follicles can also be isolated by this technique, but we have not described this because of the ease with which follicles can be obtained by the plucking of scalp hair.

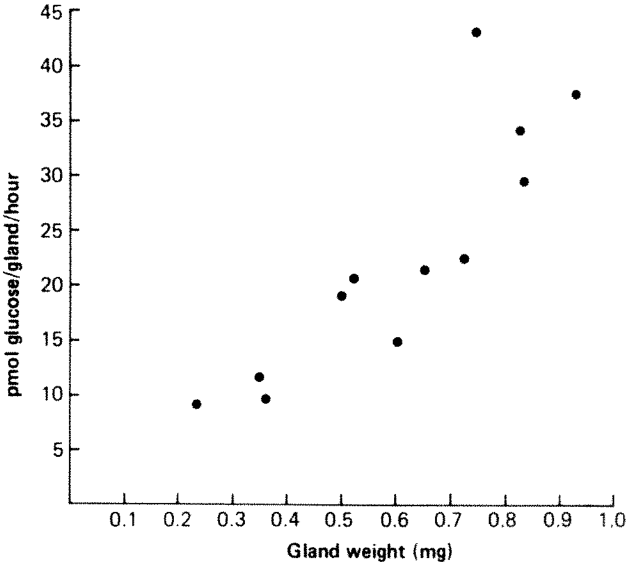

FIGURE 5. The correlation between gland wet weight and the rate of lipogenesis. Twelve glands were obtained from a 60-year-old male subject, dissected free of collagen bundles, weighed and incubated separately for 2 h in 2 mM (U-$^{14}$C) glucose as described in the text. The correlation coefficient was 0·87.

In many disease states the availability of skin may be limited to that obtained from 5-mm punch biopsies, and the shearing technique described here works when applied to such material.

The greater size of apocrine sweat glands than eccrine sweat glands is reflected in the fact that their ATP content is 3·8-fold greater than that of eccrine sweat glands (81·0 ± 12·7 pmol/eccrine sweat gland; Lee *et al.*, 1984). The ATP content of sebaceous glands is, however, only half that of apocrine glands (Table 1) despite their much larger size. This is a consequence of the fact that the bulk of the cytosol of sebaceous cells is occupied by lipid (Figs 1 & 3).

The viability of the sebaceous glands was further judged by their ability to synthesize lipid at the rate of 39·7 ± 3·7 pmol glucose incorporated into lipid/gland/h. The rate of sebaceous gland lipogenesis *in vivo* is not known. However, Cooper *et al.* (1976) have described a rate of skin plug lipogenesis of 1810 ± 3700 pmol/h/4 mm punch biopsy. The density of sebaceous glands in human skin has been reported as ranging from 100 to 900 gland/cm$^2$ (Strauss, Downing & Ebling, 1983) and this provides a range of sebaceous gland lipogenesis in skin plugs of 16·0 ± 3·3 to 144·1 ± 29·4 pmol/h/sebaceous gland. The figure we now report of 39·7 ± 3·7 pmol/h/isolated gland falls within this range.

The application of this technique of shearing has resulted in the isolation of human sebaceous and apocrine sweat glands that are viable as judged by their light and electron micrographic appearance, by their adenine nucleotide ratios and the rate of sebaceous gland lipid synthesis. We have already shown that the isolation of human eccrine sweat glands has enabled a range of metabolic studies to be performed (Kealey, 1983) and that isolated glands may be successfully maintained for up to a week (Lee *et al.*, 1984) or longer (authors' unpublished observations). It has also been shown (authors' unpublished results) that viable collecting ducts can be microdissected from eccrine sweat glands isolated by shearing, in approximately 10 min per duct and the greater size of apocrine sweat glands indicates that their microdissection would be correspondingly easier. Sebaceous gland collecting ducts, however, are not obviously exposed by isolated sebaceous glands and so their further separation by microdissection would not be apparently possible.

In conclusion, it is hoped that the availability of a simple isolation procedure for sebaceous and apocrine sweat glands will facilitate the study of their physiology and pathology.

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# Morphological changes in peripheral blood cells and skin in amiodarone-treated patients

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## SUMMARY

Amiodarone, used in the treatment of cardiac arrythmia, may lead to severe discolouration of sun-exposed skin. The lysosomal storage of a lipid-like material has been shown to be the morphological substrate for this cutaneous hyperpigmentation. Examination of peripheral white blood cells of amiodarone-treated patients disclosed identical lysosomal structures indicating that amiodarone treatment leads to a more generalized lysosomal storage of lipids and of amiodarone and its metabolites.

Amiodarone, an iodinated benzofuran derivative, has been widely used in the treatment of drug resistant cardiac arrhythmia (Podrid & Lown, 1981; Rosenbaum *et al.*, 1976; Weber *et al.*, 1984). Among a considerable number of side effects, amiodarone has been reported to cause photosensitivity and a severe progressive slate-grey hyperpigmentation of sun-exposed skin in 30% and 3%, respectively, of patients treated for prolonged periods of time (Chalmers *et al.*, 1982; Matheis, 1972). At the ultrastructural level myelin-like lysosomal structures and electron dense membrane-bound granules have been described as the morphological substrate of the cutaneous pigmentation (Korting *et al.*, 1981; Geerts, 1971).

The purpose of our study was to investigate whether amiodarone-induced ultrastructural alterations are limited to the hyperpigmented skin or whether there is involvement of other sites and organs. In this paper we describe identical lysosomal inclusions in circulating peripheral white blood cells in 82% of all patients treated with amiodarone and in 100% of those patients who developed hyperpigmentation.

## METHODS

Forty-one patients out of a total of 93 patients treated for cardiac arrhythmia with amiodarone at a maintenance dose of 200 mg/day for periods ranging from 2 weeks to 24 months were closely

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monitored for the development of hyperpigmentation or increased photosensitivity (Table 1). The remaining 52 patients were unwilling to submit to repeated examinations and were thus excluded from the study. At the time of the first examinaton seven patients had been on the drug for from 2 weeks to 2 months, eight patients for from 3 to 6 months, 11 patients for from 7 to 12 months and 15 patients for a period longer than 12 months. Fifteen patients (16%) gave a history of photosensitivity after an average duration of treatment 13 months, while four patients (4·3%) showed the classical slate grey pigmentation of the skin after an average duration of treatment greater than 20 months. One patient who had been on continuous amiodarone treatment for 36 months, was examined 12 months after withdrawal of treatment at which time his facial hyperpigmentation was still unchanged. Skin biopsies of the four patients with severe amiodarone-induced hyperpigmentation were obtained from involved facial skin and uninvolved non-exposed buttock areas. Specimens were processed for routine histopathology, stained with H&E, silver and iron stains, and also for transmission electron microscopy employing methods described previously (Konrad, Wolff & Hönigsmann, 1974).

Peripheral blood samples were obtained from 28 patients: four with hyperpigmentation and a history of photosensitivity, 11 with a history of photosensitivity only and 13 patients without photosensitivity or pigmentation. Buffy coat preparations were obtained by centrifugation of the heparinized specimens. After fixation with a formaldehyde-glutaraldehyde fixative, the specimens were processed for routine transmission electron microscopy as described previously (Konrad *et al.*, 1974), cut on a Reichert Jung Ultracut microtome and examined with a Zeiss EM9S-2 electron microscope. Peripheral blood samples from 12 healthy volunteers not on amiodarone treatment served as controls.

## RESULTS

Examination of involved hyperpigmented skin revealed changes identical with those described previously (Matheis, 1972; Korting *et al.*, 1981; Geerts, 1971; Vos *et al.*, 1972). In H&E sections aggregates of brown pigment granules were found in macrophages around blood vessels of the papillary and superficial reticular dermis, and were also seen in endothelial cells and pericytes. These pigment granules were heavily stained with silver salts, but were only weakly positive for iron. Electron microscopy revealed the granules to represent three types of structures; myelin bodies, electron-dense lysosome-like granules and transitions between the two. The greatest number of these inclusions were found in macrophages (Fig. 1) where they often filled almost the entire cytoplasm, but they were also observed in endothelial cells (Fig. 2), smooth muscle and histiocytic cells. In addition, identical structures, mostly of the myelin body type, were seen in fibroblasts, pericytes (Fig. 2), Schwann cells, axons of cutaneous nerves and to a lesser degree, within the epidermis in keratinocytes and Langerhans cells. No inclusions were found in melanocytes.

Examination of non-involved clinically normal appearing buttock skin of these patients also showed myelin body-like inclusions and dense bodies, but here they were present in much smaller numbers and were confined to endothelial cells and pericytes (Fig. 3).

Inclusions of the myelin body type were found in peripheral white blood cells of all patients with amiodarone-induced pigmentation (Fig. 4) and in 19 out of 24 patients without pigmentation (Table 1). Myelin bodies measured from 0·5 to 2·0 μm in diameter and were round to oval in shape. They consisted of multiple concentrically arranged electron dense lamellae surrounded by a unit membrane (Fig. 5). The whirls of lamellae did not exhibit a regular periodicity and, depending on the plane of section, also appeared as tubular profiles. As in the

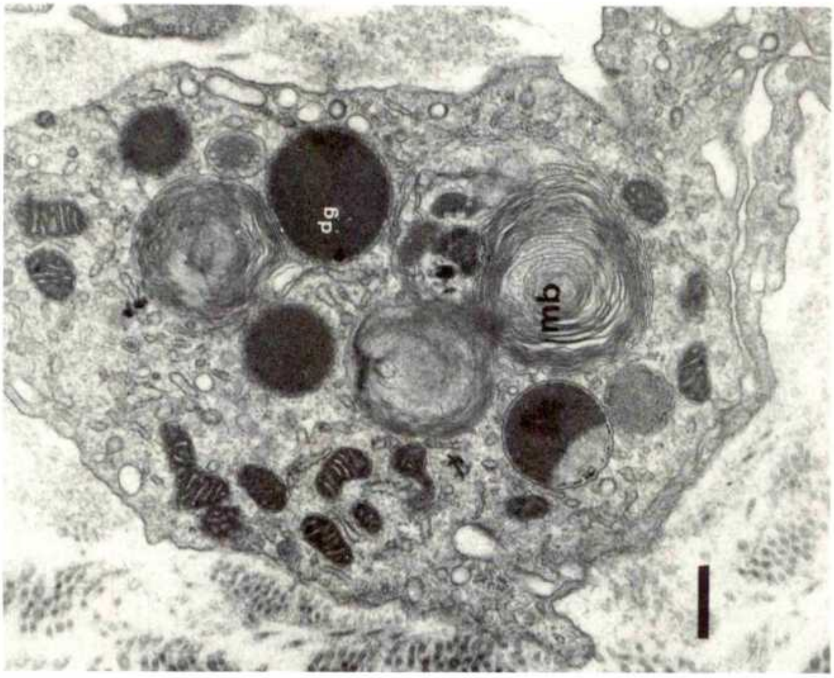

FIGURE 1. Ultrastructural changes in hyperpigmented skin. Electron dense membrane bound granules (dg) and characteristic myelin-like bodies (mb) within a perivascular macrophage. Scale bar = 1·0 μm.

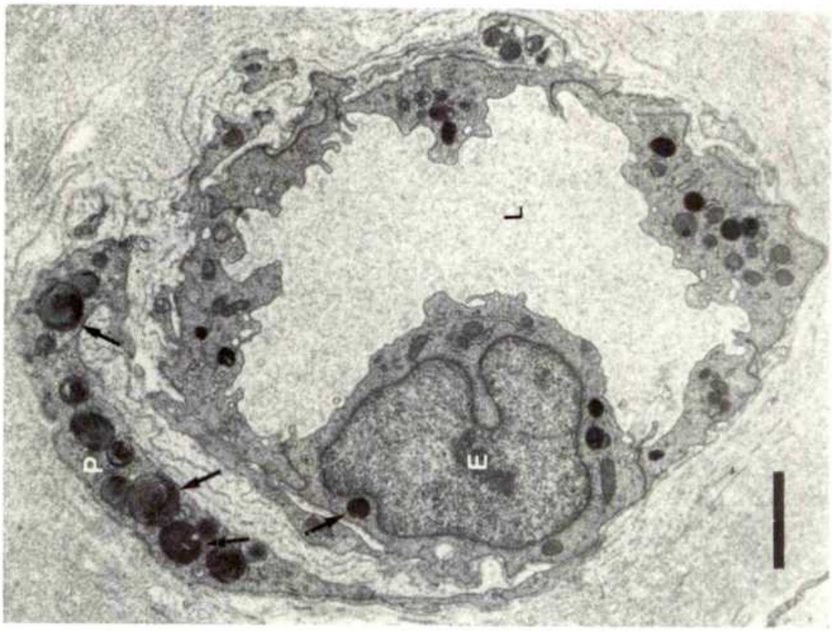

FIGURE 2. Ultrastructural changes in hyperpigmented skin. Myelin bodies and dense bodies (arrows) within endothelial cells (E) and a pericyte (P). L: vascular lumen. Scale bar = 2·0 μm.

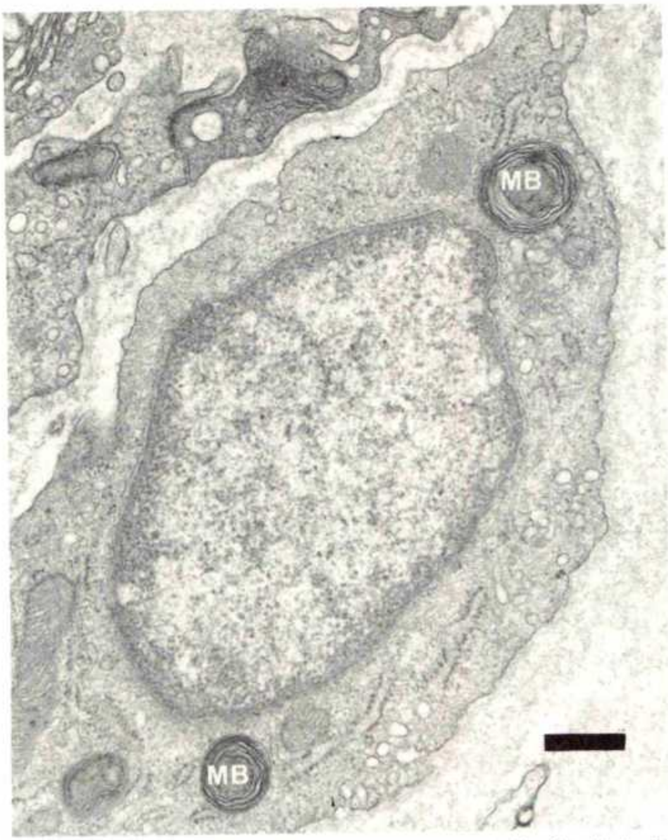

FIGURE 3. Ultrastructural changes in normal appearing non-exposed skin. Myelin bodies (MB) within a pericyte. Scale bar = 0·5 $\mu$m.

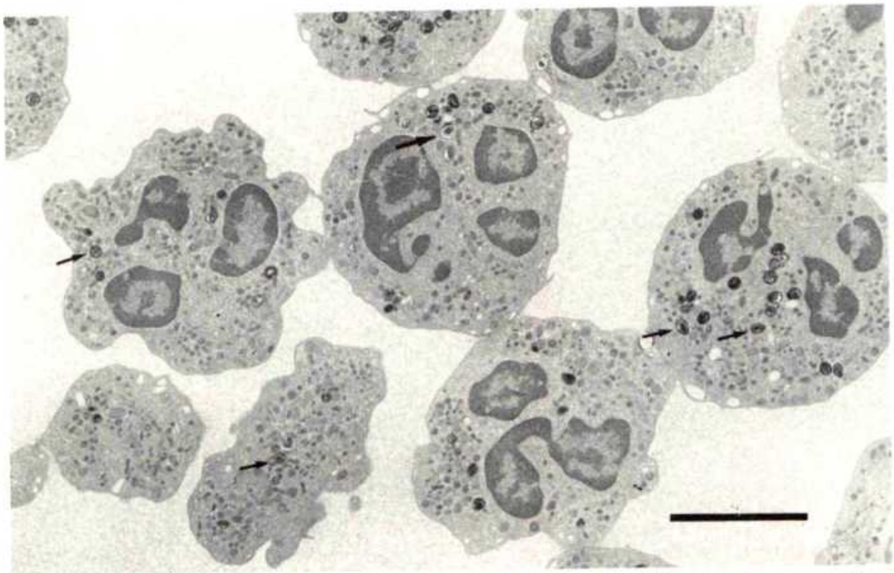

FIGURE 4. Peripheral white blood cells. Characteristic amiodarone-induced lysosomal structures (arrows) are present in most polymorphonuclear leukocytes. Scale bar = 5·0 $\mu$m.

TABLE 1. Details of subjects and tissue studies

| | | | Amiodarone-induced inclusions found in | | |
|---|---|---|---|---|---|
| | No. of patients | Duration of treatment | hyperpigmented lesional skin* | normal appearing skin† | white blood cells‡ |
| Amiodarone-treated patients with hyperpigmention | 4 | 12–24 months | 4 | 4 | 4 |
| Amiodarone-treated patients without hyperpigmentation | 24 | 2 weeks to 24 months | not done | not done | 19 |
| Healthy controls | 12 | — | not done | not done | 0 |
| Patient off amiodarone-treatment§ | 1 | 24 months | 1 | 0 | 0 |

* Myelin bodies, dense bodies and transitional-type bodies within endothelial cells, pericytes, fibroblasts, smooth muscle cells, histiocytes, Schwann cells, axons of cutaneous nerves, keratinocytes and Langerhans cells.

† Myelin bodies restricted to endothelial cells and pericytes.

‡ Myelin bodies within neutrophils (20–100%) and lymphocytes.

§ Patient, 1 year after cessation of a 24-month period of amiodarone-treatment, still heavily pigmented.

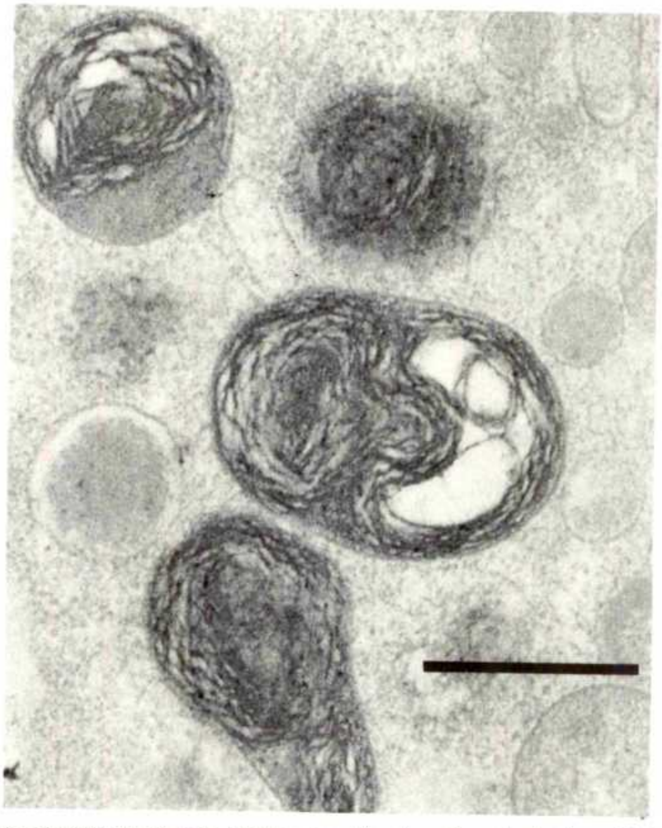

FIGURE 5. Peripheral white blood cells. Higher magnification of myelin bodies within a polymorphonuclear leukocyte. Scale bar = 0·5 μm.

skin specimens, an electron dense core was sometimes surrounded by loosely packed lamellae indicating transition into dense body type lysosomes.

The number of inclusions was highest in neutrophils and although exact quantification was not attempted the incidence of cells affected ranged from 20 to 100%, which correlated with the duration of treatment. Within the first 2 weeks of amiodarone treatment no inclusions were found; between the 3rd and 6th week of treatment cells with inclusions were encountered sporadically but with longer treatment times of up to 6 months their incidence increased to 20–50%. The largest numbers of cells with inclusions (80–100%) were found in patients on long-term amiodarone treatment of over 12 months. Lymphocytes also contained inclusions, although in lower numbers, but eosinophils and basophils were devoid of them.

Of 28 samples examined, 23 were positive for inclusions (Table 1). These included four patients with cutaneous pigmentation and 19 patients without pigmentary changes. All of these patients had been on a continuous amiodarone treatment for 6–24 months. Five blood samples failed to show myelin-body-like inclusions and these were from patients with a duration of amiodarone treatment of less than 6 weeks. The 12 control blood samples of healthy volunteers who did not receive amiodarone failed to reveal myelin bodies within leukocytes.

One heavily pigmented patient who had been on continuous amiodarone treatment for 24 months and at this time had shown myelin bodies in 90% of his peripheral neutrophils was re-examined 12 months after termination of amiodarone treatment. At this time no inclusions were found in his peripheral white blood cells and non-exposed skin although clinical pigmentation was unchanged and inclusions were still present in his hyperpigmented skin.

## DISCUSSION

Reported side effects of amiodarone therapy (Weber *et al.*, 1984; Harris *et al.*, 1983) include life-threatening pulmonary fibrosis, pneumonitis (Sobol & Rakita, 1982; Marchlinski *et al.*, 1982; Zaher *et al.*, 1983), hepatotoxicity (Simon *et al.*, 1984), disturbance of thyroid function (Stäubli *et al.*, 1981), peripheral neuropathy (Pellissier *et al.*, 1984) and yellow-brown stippling of the cornea (verticillate keratopathy) (D'Amico, Kenyon & Ruskin, 1981). Long-term, high dose therapy with amiodarone leads to photosensitivity in 30% of the patients and in approximately 3% it is associated with a progressive slate-grey pigmentation of sun-exposed skin (Waller, Bradner & Curtis, 1984; Zachary *et al.*, 1984; Miller & McDonald, 1984; Trimble *et al.*, 1983). Recent studies have established that the morphological substrate of amiodarone-induced pigmentation are the myelin body- and dense body-types of lysosomal granules also described in the present study (Geerts, 1971; Zachary *et al.*, 1984; Miller & McDonald, 1984; Trimble *et al.*, 1983). Histochemical studies have revealed that the granules contain lipids (Matheis, 1972), and electron-probe X-ray analysis and high performance liquid chromatography have established that they contain iron, sulphur, iodine, amiodarone and its major metabolite, desethylamiodarone (Zachary *et al.*, 1984; Trimble *et al.*, 1983). It has also been shown that pigmented skin contains markedly higher concentrations of both amiodarone and desethylamiodarone as compared to unaffected skin (Holt *et al.*, 1984). This suggests that amiodarone and its metabolites concentrate in lysosomes causing an accumulation of lipids, similar to what occurs with neutral glycosphingolipids in Fabry's disease (Lüllmann, Lüllmann-Rauch & Wassermann, 1978; Faraggiana *et al.*, 1981). The similarity of the amiodarone-induced myelin bodies to the fingerprint granules in Fabry's disease has suggested to some that amiodarone pigmentation represents a drug-induced lipid storage disease with corneal and cutaneous manifestations (Trimble *et al.*, 1983).

The fact that a significant number of patients on amiodarone are photosensitive and that amiodarone-induced pigmentation occurs only in sun-exposed sites suggests that light energy is involved in precipitating myelin body formation and lipid storage. This present study shows that identical structures also occur not only in non-exposed skin sites of amiodarone-treated patients, but also in the peripheral white blood cells of almost 100% of patients who have been on amiodarone therapy for longer than 6 months. This suggests that the effects of amiodarone are more universal than originally assumed and that the compound induces a generalized derangement of lysosomal structure and, possibly, function. This is in accordance with the findings of Holt *et al.* (1984) and with the results of animal studies which have shown that cationic amphiphilic drugs lead to an accumulation of polar lipids within lysosomes throughout the body (Lullmann *et al.*, 1978).

At present we do not know whether the lysosomal inclusions in the peripheral blood cells have a functional significance, whether they lead to clinical symptoms and whether the described pulmonary and hepatic complications of amiodarone treatment are the result of identical lysosomal changes in these organs. The recently described amiodarone-induced peripheral neuropathy (Pellissier *et al.*, 1984) where identical inclusions were found in Schwann cells—as was the case in the present study—appears to confirm the concept of an amiodarone-induced generalized lysosomal storage disease.

These observations do not explain the pigmentary changes seen in some patients. In this context it should be noted that whereas most amiodarone-induced inclusions in non-exposed skin and in peripheral leucocytes are of the myelin body type, the majority of inclusions within macrophages of exposed skin are of the dense granular type. It is our working hypothesis that the latter evolve from amiodarone-induced myelin bodies in the course of multiple, possibly subclinical phototoxic reactions.

## ADDENDUM

During the processing of this manuscript Dake *et al.* (1985) have described similar lysosomal inclusion bodies in lung, liver, lymph nodes and blood leukocytes of patients with pulmonary amiodarone toxicity.

## ACKNOWLEDGMENT

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# Effects of ultraviolet radiation and immunosuppressive therapy on mouse epidermal cell kinetics

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## SUMMARY

The effects of immunosuppressive drugs on epidermal cell mitotic activity and the proliferative response of epidermis following ultraviolet radiation (UVR) were tested. Hairless (Skh-hr 1) mice were treated with immunosuppressive drugs at equivalent clinical doses with or without concomitant UVR (290–400 nm). Epidermal parameters measured were mitotic index ($I_m$), rate of entry into mitosis ($F_m$), flash labelling index (FLI), rate of entry into DNA synthesis ($F_s$) and DNA content (flow cytometric analysis). In non-irradiated skin, prednisolone therapy depressed both mitotic activity and DNA synthesis; azathioprine and cyclosporin A had no effect; cyclophosphamide therapy increased the $F_m$ and FLI values. Following repeated doses of UVR, there were enhanced mitotic activity and DNA synthesis in epidermis. Prednisolone therapy moderately depressed both proliferative responses; cyclophosphamide enhanced mitotic activity; azathioprine and cyclosporin A had no effect on these responses. The significance of these findings in relation to potential for increased susceptibility of skin to UV-induced carcinogenesis is discussed.

Kidney transplant recipients receiving the standard immuno-suppressive regime of azathioprine and steroids have increased susceptibility to skin cancer. The incidence of skin cancer in these patients varies between countries but is highest in areas of high sun exposure. In Australia, which is regarded as a 'high risk' area for all skin cancers, approximately 30% of transplant patients will develop skin cancer by 5 years post-transplant (Sheil, 1984; Hardie *et al.*, 1980) and in these patients the cancers occur predominantly on sun-exposed areas of the body (Hardie *et al.*, 1980).

Sun-exposure and racial origin are clearly important predisposing factors in human skin carcinogenesis but the additional predisposing factors in transplant recipients are unknown.

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Depressed immunosurveillance and activation of latent oncogenic virus infection as consequences of immunosuppressive therapy have been postulated (Sheil, 1983). In addition to immune suppression, the anti-metabolite actions of currently-used immune suppressive agents must be considered. While the anti-mitotic effect of corticosteroids on epidermal cells is well recognized (Fisher & Maibach, 1971; Wilson Jones, 1976), the effect of the anti-metabolite azathioprine on these cells has not been described.

The purine-antagonist action of azathioprine suggests that it has the potential to inhibit keratinocyte mitotic activity, and indeed azathioprine has been used successfully in clinical practice for the treatment of psoriasis, a condition characterized by a rapid turn-over of epidermal cells (Du Vivier, Munro & Verbov, 1974).

Following the exposure of skin to ultraviolet radiation (UVR), including sunlight, there is normally a mild proliferative cellular response within the germinal layer of the epidermis. In the presence of azathioprine and prednisolone therapy, this response might be expected to be depressed. Any interference with the normal repair or replacement of skin cells damaged by UVR could be an important contributing factor to UV-induced skin carcinogenesis.

In this study we have used a hairless mouse model to investigate the effect of four commonly used clinical immunosuppressants on murine epidermal cell kinetics. Mitotic activity was assessed by the three techniques of (a) direct counts of mitotic figures, (b) autoradiographic determination of scheduled DNA replication, and (c) flow cytometry.

## METHODS

### *Animals*

Hairless, albino, Skh-hr 1 mice were used in all experiments. These were inbred, female and between 8–14 weeks old. Animals were held under constant conditions of temperature (22°C) and humidity (50%). There was a 12:12 h light:dark cycle and light was provided by a General Electric Gold fluorescent light. The diet was a standard, commercial mouse diet supplied *ad libitum*. The care and housing of the animals conformed to guidelines for the use of laboratory animals established by the National Health and Medical Research Council of Australia.

### *Immunosuppressive drug therapy*

The dosages of the immunosuppressants used have already been reported (Kelly *et al.*, 1985) to be equivalent to maintenance doses of the same drugs in human transplant recipients when estimated on the basis of body surface area and immunosuppressive and toxicological effects. Azathioprine ('Imuran', Wellcome) was dissolved in distilled water and administered IP at a dosage of 15 $\mu$g/g body weight; prednisolone (Glaxo) in saline, IP, 20 $\mu$g/g; cyclophosphamide ('Endoxan', Bristol) in distilled water, IP, 15 $\mu$g/g; cyclosporin A (Sandoz), in peanut oil, per os, 60 $\mu$g/g. Solutions of azathioprine and cyclophosphamide were made up fresh daily, immediately before use: Solutions of prednisolone and cyclosporin A were made fresh weekly. All drugs were given as a 0·1 ml volume. Control mice received isotonic saline.

### *Ultraviolet radiation*

The UV radiation source was a bank of three Sylvania F40/350 BL tubes emitting in the UV-A spectrum, and one Oliphant FL40SE tube emitting in the UV-B spectrum. At a target distance of 20 cm, the irradiance was 1·72 mW/cm$^2$ at a wave length of 320–380 nm, and 0·20 mW/cm$^2$ at a wave length of 313 nm. The minimal erythemal dose time was determined visually to be 5 min. During UVR the mice were unrestrained and conscious within boxes.

### *Histology*

Six mm punch biopsies were taken under ether anaesthesia from the skin over the dorsum. These were fixed in 4% buffered formalin, embedded in paraffin and 6 $\mu$m thick sections prepared. After staining with haemotoxylin and eosin, sections were examined under oil immersion for mitotic figures or nuclear grains. In each case, 2000 germinative cells were scanned; only interfollicular regions were scored and several different areas of each section were examined.

### *Mitotic figures*

Cells in prophase, metaphase, anaphase and telophase, were counted. The percentage incidence of mitotic figures in the germinal layer of the epidermis was regarded as the mitotic index ($I_m$).

The rate of entry of epidermal cells into mitosis ($F_m$) was determined following the injection of drugs to induce metaphase arrest (Duffill *et al.*, 1977). 5 $\mu$g of vincristine sulphate (Sigma) and 10 $\mu$g of vinblastine sulphate (Sigma) were injected IP into mice. Skin biopsies for enumeration of metaphase figures were collected immediately prior to injection and 2 h post-injection. The number of metaphases in 2000 germinative cells was counted in both samples and the rate of accumulation of metaphases per $10^3$ germinative cells per h ($F_m$) determined.

### *Flash labelling index (FLI)*

FLI was determined following an IP injection of 50 $\mu$Ci of [$^3$H]-thymidine (20 Ci/mM; Amersham) into mice. The dorsal epidermis was biopsied one hour later and autoradiograms prepared using Ilford K5 emulsion with exposure at 5°C for 10 days. Cells with three or more grains over the nucleus were counted; cells were scored as lightly-labelled (3–15 grains) or heavily-labelled (more than 20 grains). The FLI was calculated as the percentage incidence of germinative cells with labelled nuclei.

The rate of entry of epidermal cells into DNA synthesis ($F_s$) was determined as follows: 10 $\mu$Ci of [$^3$H]-thymidine in a 50 $\mu$l volume was injected intradermally into each of two sites on the dorsal epidermis. One hour later, one site was biopsied while the second site received a further injection of 10 $\mu$Ci of [$^3$ H]-thymidine; this second site was biopsied after a further hour. The skin biopsies were then processed and assessed for FLI. The differences between the 1 h and 2 h FLI values yields the number of cells entering DNA synthesis per $10^3$ germinative cells per hour ($F_s$).

### *Flow cytometry*

Basal and differentiating cells were separated from epidermis by trypsinization (Clausen *et al.*, 1976). These cell samples were mechanically disaggregated in a staining solution containing propidium iodide 50 $\mu$g/ml (Calbiochem), RNAase A grade 1 mg/ml (Sigma) and Triton-X-100 1% v/v (Packard) in 0·9% saline. The sample was filtered through 45 $\mu$m nominal pore size nylon gauze, a cell count performed and the cell concentration adjusted to $1 \times 10^6$ per ml with staining solution. The DNA content of this suspension was measured using a 50H cell sorter interfaced to a 2150 computer (Ortho Instruments). Determination was then made of the number of resting ($G_1$) cells with normal diploid (2N) DNA content, a minor peak of cells in $G_2$ or early mitosis (M) with a 4N DNA content, and cells in the process of synthesizing DNA (S) with DNA values between 2N and 4N. Between $2$–$10 \times 10^4$ cells were examined per sample.

### *Research plan*

The diurnal variation of mitotic activity in the epidermis was assessed in two groups of 10 mice held in separate rooms. One group was exposed to the light phase between 0700 and 1900 h and the second group between 1900 and 0700 h. After 4 weeks in these environments, 4 mm punch biopsies of skin were collected from each animal every 4 h over a 24 h period. The $I_m$ was then determined for each biopsy.

To determine the effect of immunosuppressive therapy on $I_m$, FLI, $F_m$ and $F_s$, immunosuppressive drugs were given daily with or without 7 min UVR for 5 consecutive days immediately prior to the day of assay; drug therapy was given between 0800 and 0900 h, and UVR between 0900 and 1000 h. On the day of assay, mice received immunosuppressive drug therapy but no UVR. Skin biopsies were collected between 1200 and 1500 h. Assays for $I_m$ and $F_m$ were carried out on the same mice. Assays for FLI and $F_s$ were each performed in different mice. With each assay there were between five and eight mice per treatment group.

The influence of UVR on FLI was not studied because of the complication of UVR-induced, unscheduled DNA synthesis in the epidermal cell autoradiographs.

For the flow cytometry assay, groups of 24 mice were treated with immunosuppressive drugs for 7 consecutive days. On the fourth day of treatment the mice received 7 min UVR. Six mice from each treatment group were then sacrificed each day for 4 days. The skin over the dorsum and flanks was removed after death and the basal epidermal cells isolated. Cells from three mice within each treatment group were pooled for flow cytometric analysis so that two samples per treatment group per day were analysed.

### *Statistical analyses*

The results of the flow cytometric study were analysed with Duncan's multiple range assay. The results of all other studies were analysed with the Student's *t*-test.

## RESULTS

### *Diurnal variation*

Figure 1 shows the variation in the mitotic activity of keratinocytes over a 24 h period and the influence of the time of onset of the 12 h light phase on this phenomenon. When the light phase was between 0700 and 1900 h, peak mitotic activity occurred at 0600 h and minimal activity at 1400 h. When the light/dark cycle was reversed, peak activity then was at 1800 h and minimal activity at 1000 h.

For all ensuing assays in this study, a reversed light/dark cycle with light beginning at 1900 h was used.

### *Mitotic index ($I_m$)*

The data are presented in Table 1. Therapy with azathioprine, cyclophosphamide and cyclosporin A had no effect on $I_m$, while prednisolone either alone ($P < 0.05$) or in combination with azathioprine ($P < 0.01$) or cyclosporin A ($P < 0.05$) significantly depressed $I_m$.

Following repeated doses of UVR, there was a mean 31% increase in $I_m$ in control animals. Treatment with azathioprine, cyclophosphamide or cyclosporin A had no effect on this response; treatment with prednisolone either alone ($P < 0.01$) or in combinaton with azathioprine ($P < 0.01$) or cyclosporin A ($P < 0.01$) depressed the response compared to control mice, but not to the same degree as in mice not receiving UVR.

### *Flash labelling index (FLI)*

The results of this study are shown in Table 2. In control mice the mean FLI was 10.76%; of

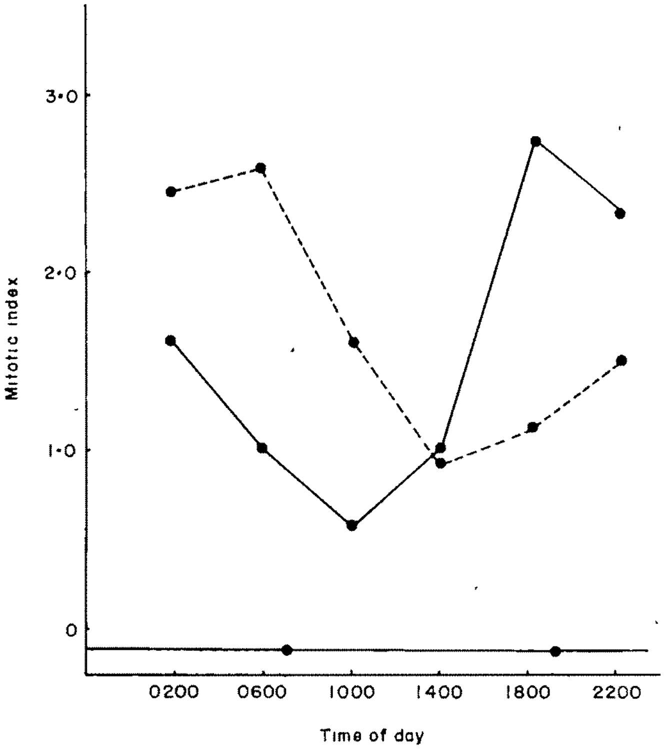

FIGURE 1. Diurnal variation in the mitotic activity of mouse epidermis. ●——● Light phase 1900–0700 h; ●- - - - - -● Light phase 0700–1900 h.

TABLE 1. Mitotic activity ($I_m$) in epidermis of immunosuppressed mice with and without UVR

| | | Mitotic index (mean ± sd) | |
|---|---|---|---|
| Treatment | No of mice tested | No UVR | UVR |
| Saline | 7 | 1·39 ± 0·30 | 1·82 ± 0·88 |
| Azathioprine | 8 | 1·45 ± 0·32 | 1·73 ± 0·69 |
| Prednisolone | 8 | 0·63 ± 0·29* | 1·01 ± 0·72* |
| Azathioprine/ prednisolone | 7 | 0·50 ± 0·16* | 0·83 ± 0·54* |
| Cyclophosphamide | 8 | 1·37 ± 0·62 | 1·91 ± 0·48 |
| Cyclosporin A | 5 | 1·33 ± 0·45 | 1·79 ± 0·81 |
| Cyclosporin A/ prednisolone | 6 | 0·67 ± 0·22 | 1·18 ± 0·73* |

* Significantly different from result with saline control, determined by Student's *t*-test.

TABLE 2. Flash labelling index (FLI) in epidermis of immunosuppressed mice

| | Labelling index (mean ± sd) | |
|---|---|---|
| Treatment | < 15 grains nucleus | Total labelled cells |
| Saline | 2·99 ± 0·82 | 10·76 ± 3·08 |
| Azathioprine | 2·48 ± 0·71 | 11·83 ± 2·08 |
| Prednisolone | 1·57 ± 0·71 | 6·93 ± 2·56* |
| Azathioprine/ prednisolone | 2·64 ± 0·61 | 6·47 ± 2·60* |
| Cyclophosphamide | 3·80 ± 0·77 | 14·33 ± 1·82* |
| Cyclosporin A | 2·66 ± 0·48 | 11·43 ± 2·14 |
| Cyclosporin A/ prednisolone | 1·48 ± 0·84 | 7·00 ± 2·20* |

* Significantly different from saline control value by Student's *t*-test

these cells, a mean of 2·9% had between 3 and 15 grains per nucleus. Treatment with either azathioprine or cyclosporin A had no effect on FLI. Cyclophosphamide had a stimulatory effect ($P < 0·05$), while prednisolone either alone or in combination with azathioprine or cyclosporin A had a depressive effect ($P < 0·05$). None of the immunosuppressive treatment regimes significantly affected the absolute numbers of lightly-labelled (3–15 grains per nucleus) cells in the epidermis.

### *Influx of cells into mitosis ($F_m$) and DNA synthesis ($F_s$)*

These data are presented in Table 3. In control mice, $F_m$ was a mean of 4·2 cells/10³ cells/h and $F_s$ was 5·7 cells/10³ cells/h. In control mice exposed to repeated UVR, the mean $F_m$ was almost doubled to 8·1. Treatment with azathioprine and cyclosporin A had no effect on these

TABLE 3. Rate of entry of epidermal cells into mitosis ($F_m$) and DNA synthesis ($F_s$)

| | $F_m$† | | | $F_s$† | |
|---|---|---|---|---|---|
| Treatment | No. of mice | No UVR | UVR | No. of mice | No UVR |
| Saline | 7 | 4·2 ± 2·1 | 8·2 ± 3·9 | 8 | 5·7 ± 2·5 |
| Azathioprine | 8 | 3·9 ± 1·8 | 8·1 ± 4·1 | 8 | 5·8 ± 2·2 |
| Prednisolone | 8 | 2·0 ± 0·6* | 2·9 ± 1·9* | 8 | 2·0 ± 0·8* |
| Azathioprine/ prednisolone | 7 | 2·6 ± 1·2* | 3·3 ± 2·4* | 7 | 2·9 ± 0·7* |
| Cyclophosphamide | 8 | 5·3 ± 2·8 | 8·5 ± 4·8 | 8 | 8·5 ± 3·4* |
| Cyclosporin A | 5 | 3·9 ± 2·1 | 7·8 ± 3·3 | 8 | 5·3 ± 1·9 |
| Cyclosporin A/ prednisolone | 6 | 3·1 ± 1·9 | 5·5 ± 3·2* | 8 | 3·7 ± 2·4* |

† Number of cells entering mitosis ($F_m$) or DNA synthesis ($F_s$) per 10³ germinative cells per hour.

* Significantly different from result with saline control, determined by Student's *t*-test.

TABLE 4. Flow cytometric analaysis of epidermal cells following UVR

| | S phase | | | | $G_2$ + M phase | | | |
|---|---|---|---|---|---|---|---|---|
| | Hours post-UVR | | | | Hours post-UVR | | | |
| Treatment | 0 | 24 | 48 | 72 | 0 | 24 | 48 | 72 |
| Saline | 7 3 | 14 0 | 18 0 | 9 0 | 5·2 | 3 3 | 5 3 | 5 0 |
| Azathioprine | 6 8 | 12 1 | 14 6 | 9·1 | 6 5 | 3·1 | 5 6 | 5·0 |
| Prednisolone | 6 8 | 11·3 | 12 4 | 7 6 | 5 0 | 3·0 | 3 3 | 5 3 |
| Azathioprine/ prednisolone | 8·0 | 12·0 | 13 0 | 9·1 | 5 5 | 6 1 | 3·6 | 6 2 |
| Cyclophosphamide | 6 9 | 10·5 | 25 1 | 8 2 | 4 8 | 4 5 | 11 7 | 6·0 |
| Cyclosporin A | 6 4 | 10 3 | 17·3 | 7 3 | 4 7 | 4·3 | 6 5 | 6 1 |
| Cyclosporin A/ prednisolone | 5 7 | 11·3 | 13 1 | 6 9 | 5·5 | 5 1 | 5 3 | 4·9 |

Values presented are the means of duplicate samples; each sample was cells pooled from three mice. The coefficient of variation of the $G_1$ peak was 3 26 ± 0 58% (mean ± sd).

parameters with or without UVR. Treatment with prednisolone either alone or in combination with azathioprine or cyclosporin A significantly ($P < 0\cdot05$) depressed both the $F_m$ value with or without concomitant UVR and the $F_s$ value. Cyclophosphamide therapy had a slight but significant ($P < 0\cdot05$) stimulatory effect on $F_s$.

*Flow cytometry*

The results of this assay are contained in Table 4. With all seven treatment groups there was a shift in the proportion of cells in S phase following UVR. There was an increase in S phase cells by 24 h post-UVR, peaking at 48 h and returning almost to pre-UVR levels at 72 h. There were insufficient numbers of replicates to test the significance of these shifts within each treatment group, but when the results of all treatments were pooled for analysis, the increases in the proportions of S phase cells at 24 and 48 h post-UVR were significant ($P < 0\ 01$ and $P < 0\cdot005$ respectively).

In control (saline treated) mice, there was a small drop in the proportion of cells in $G_2$ + M phase at 24 h following UVR but the proportion of these cells had returned to the pre-UVR level at 48 h post-UVR. A similar finding was recorded in mice treated with azathioprine or prednisolone although in the latter group the proportion of $G_2$ + M phase cells was still depressed at 48 h post-UVR. In mice treated with cyclophosphamide, there was a substantial increase in the proportion of these cells at 48 h post-UVR. However, when the results of all treated groups were pooled, there were no significant differences in the proportion of cells in $G_2$ + M phase with time following UVR ($P > 0\cdot05$).

When results between treatment groups were analysed, there were no significant differences in the numbers of cells in either S phase or $G_2$ + M phase prior to UVR ($P > 0\cdot05$). However, at 48 h post-UVR there was a significant depression in S phase cells in mice treated with either prednisolone ($P < 0\cdot05$) or an azathioprine/prednisolone combination ($P < 0\cdot005$) and an increase in the proportion of cells in both S phase ($P < 0\cdot005$) and $G_2$ + M phase ($P < 0\cdot001$) in mice treated with cyclophosphamide.

## DISCUSSION

Mammalian epidermis has a rapid cell turnover. The maintenance of equilibrium between cell loss and cell replacement is not well understood. Tissue-specific growth inhibitors, termed 'chalones', are believed to play an important role probably acting via a negative feedback mechanism (Bullough, 1972) by controlling the rate at which epidermal cells move through the $G_1$, S, $G_2$ and M phases of the cell cycle (Elgjo, Laerum & Edgehill, 1971; Elgjo, Clausen & Thorud, 1981). In addition, a number of hormones or hormone-like substances, such as glucocorticoids, catecholamines, adrenaline and cyclic nucleotides, are able to modify cell mitotic activity. Whether these act indirectly, through agents such as chalones, or directly upon epidermal cells, is not known, but a direct action does seem possible since keratinocytes are known to have specific receptor sites for glucocorticoids (Smith & Shuster, 1984).

The epidermis of animals and man poliferates at an inconstant rate with a basic circadian rhythm which correlates with phases of bodily activity and rest. In man, epidermal mitotic activity is highest at night and lowest during the day (Fisher, 1968; Schell *et al.*, 1981), correlating inversely with blood levels of corticosteroids and catecholamines which rise during activity; it is generally assumed that blood levels of such substances are directly linked to the regulation of epidermal activity (Bullough, 1948; Bullough & Lawrence, 1968; Schell, Hornstein & Schwarz, 1980). With nocturnal animals such as mice, a reversal of this situation with a peak epidermal proliferative activity occurring with rest during the hours of light might be expected. Although this subject has been studied extensively in rodents, the results of the different studies vary considerably (*see* Potten, 1975, for review). Likely reasons for this variability include differences in the housing and handling conditions for the animals. Usually maximum disturbance from routine husbandry procedures occurs during daylight hours, which together with handling during the experiments would seriously disrupt any diurnal cycle. In this study, these disturbances were minimized by using a reversed day/night cycle. In this way, a diurnal cycle was observed with a peak and trough of epidermal activity occuring during the light and dark phases respectively.

In studies of the effects of drugs on cell cycle kinetics the influence of diurnal variation in cell activity could be important. The time in the diurnal cycle at which the cells are examined for any resulting effects could have a significant influence on the results. With the methods used here of a reversed light/dark cycle, procedures such as UVR and drug administration were performed shortly after the onset of the dark phase so that the maximum effect was obtained at the time of lowest level of epidermal cell mitotic activity. There were two reasons for this experimental design. One was the suggestion that inhibitors of epidermal cell proliferation are more effective when applied during the lowest rate of cell proliferation (Elgjo *et al.*, 1981). The other was to simulate clinical practice. Renal transplant patients normally take their immunosuppressive medication in the morning and so receive exposure to sunlight during times of maximum blood levels of the drugs and minimal epidermal proliferation.

In the mouse model used here, the epidermal cells from the dorsal skin showed a mean $I_m$ of 1·39%, FLI of 10·76%, $F_m$ of 4·2 cells per $10^3$ cells per h and $F_s$ of 5·7 cells per $10^3$ cells per h. These values are in general agreement with those obtained by others (Potten, 1975). In the hairless mouse, the duration of the S and M phases in epidermal cells are estimated to be approximately 6 and 1 h respectively (Potten, 1975), hence the discrepancy between $I_m$ and FLI values. The slightly higher rate of influx of epidermal cells into DNA synthesis compared to influx into mitosis is anticipated for epidermis (Duffill *et al.*, 1977).

Exposure of skin to UVR induced a proliferative response within the epidermis. The surge in

DNA activity peaked at 48 h post-UVR at which time there was a 147% increase in the proportion of cells in S phase, followed by a rapid return to the resting rate. However, there was no detectable corresponding increase in the proportion of cells in $G_2$ and M phases, and taken with the finding of a 31% increase in the $I_m$ in skin exposed to repeated UVR, it seems likely that a proportion of the S phase cells are either delayed or prevented from completing mitosis. The possibility that UVR may extend the $G_1$ and $G_2$ resting phases by direct depression of RNA and protein synthesis has been suggested by the work of others (Fukuyama, Epstein & Epstein, 1967; Epstein, Fukuyama & Fye, 1970). An alternative explanation for this phenomenon is the induction of chalone production as a result of UVR-induced cell proliferation or cell damage. Various chalones have been isolated from mouse epidermis which inhibit different stages of the cell cycle, and specific inhibition at the S-$G_2$ transition has been demonstrated (Elgjo *et al.*, 1981).

The major objective of this study was to determine the effect of immunosuppressive therapy on epidermal cell kinetics. The results show that two immunosuppressants commonly used in clinical transplantation, prednisolone and cyclophosphamide, do modify the mitotic activity of mouse epidermal cells, with both the normal mitotic cycle and the proliferative response to UVR being affected. Neither azathioprine nor cyclosporin A therapy had any such effects. Therapy with prednisolone reduced both DNA synthesis and mitotic activity of epidermal cells in unmodified skin. The proliferative response of epidermis following UVB was also reduced by prednisolone therapy. This inhibitory effect of prednisolone is most likely to be operating through a direct receptor binding on the surface of epidermal cells. Steroid receptor complexes exert a variety of intracellular effects including interaction with nuclear DNA, inhibition of glucose uptake and potentiation of cyclic AMP (Whitfield, McManus & Rixun, 1970; Baxter & Forsham, 1972). Despite the anti-mitotic effect seen with prednisolone, this drug still allowed a qualitative proliferative response to UVR, and so has uncertain importance in terms of promotion of UVR-induced carcinogenesis. It is also possible that prednisolone therapy may act indirectly through thinning of the epidermis as a consequence of depressed epidermal cell mitosis and replacement. Such thinning of the epidermis could be expected to permit greater penetration through the skin of UV light, particularly the carcinogenic UV-B wavelength. Increased exposure to UV-B of dermal components, germinal epidermal cells and Langerhans cells might predispose to cancer.

Cyclophosphamide therapy had no apparent effect on mitotic activity of mouse epidermis, but somewhat surprisingly had a stimulatory effect on epidermal DNA synthesis. Cyclophosphamide, an alkylating agent, induces DNA damage selectively in dividing cell populations and this usually leads to cell death (Crawthorn & Roberts, 1966; Strauss, Coyle & Robbins, 1969). Some increase in the level of unscheduled DNA synthesis in association with repair processes might be expected to follow the drug-induced DNA damage, but this would not appear to be an important source of the increased DNA synthesis seen here because (a) the incidence of lightly-grained nuclei in the autoradiograms, generally accepted as an index of DNA excision-repair activity (Brenner & Gschnait, 1979), was only slightly higher in cyclophosphamide-treated mice than in control mice, and (b) flow cytometry revealed a large increase in the proportion of S phase cells in cyclophosphamide-treated epidermis following UVR. It is interesting in this respect to note that lymphocytes from patients treated with chlorambucil, another alkylating agent, show increased DNA synthesis (Hill, 1972). It would appear that while there is an increased proliferative effect in the epidermis of cyclophosphamide-treated mice, not all cells undergoing replication progress past S phase. The reason for the increased cellular proliferation is not clear, but it may be due to a normal proliferative response to drug-induced cell toxicity.

Inhibition of the transition from S to $G_2$ phase may be a direct consequence of the anti-metabolite effect of cyclophosphamide on the genome.

The negative finding here with azathioprine is unexpected. As a purine antagonist, azathioprine prevents cells in $G_1$ from entering S phase, although cells already in S phase are unaffected (Wheeler, Bowdon & Adamson, 1972). It is also generally assumed that azathioprine acts non-specifically on proliferating cells, so that epidermis as a rapidly dividing tissue might reasonably have been expected to be preferentially affected by azathioprine, particularly as fingernail growth is reported to be retarded in azathioprine-treated patients (Dawber, 1970). However, it seems that azathioprine exerts a differential effect on the mitotic activity of various rapidly dividing, non-lymphoid tissues. For example, in azathioprine-treated patients, depression of haemopoietic tissue proliferation is well recognized, while other rapidly dividing tissues such as gut epithelium and gonads show no evidence of mitotic inhibition (Phadke, MacKinnon & Dossetor, 1970; Egger & Ackman, 1971). Whether this apparent sparing of certain tissues, including mouse epidermis as suggested in this study, is a result of differing tissue specificities or is a dose-related effect is unknown. The finding of a lack of an inhibitory effect of azathioprine on mouse epidermis is at odds with the reported clinical success of azathioprine therapy in treating the hyperproliferative skin disorder, psoriasis (Du Vivier *et al.*, 1974). While it has been generally assumed that this results at least in part from direct inhibition of epidermal cell proliferation, there is no clinical or laboratory evidence that this is the case and the results of this study suggest that there may be an alternative mechanism of action. A species difference in the response to azathioprine may be an explanation, as there are differences between the two species in the metabolism of the parent azathioprine compound; even so, the mechanism of the ultimate immunosuppressive effect of azathioprine on the genome appears to be similar in the two species (Elion & Hitchings, 1965).

The lack of any anti-mitotic effect with cyclosporin A is not surprising as this agent affects predominantly certain lymphoid cell subsets and is generally sparing on non-lymphoid tissues (Thompson, 1983). Nevertheless, hirsutism is a feature of cyclosporin A therapy in both mice and humans, reportedly as a result of enhanced keratinization (Calne, Rolles & White, 1981; Pendry & Alexander, 1982). While it is possible that cyclosporin A induces enhanced proliferation within hair follicles, the results of this study suggest that the mitotic activity of epidermis is unaffected. This conclusion is further supported by the finding that in rats, cyclosporin A therapy had no adverse effect on the normal rate of wound closure and epithelization (Eisinger & Sheil, 1984).

The object of this study was to assay the effects of immunosuppressive agents on epidermal cell mitotic activity to determine whether these effects might predispose to carcinogenesis in UVR-exposed skin. In clinical transplantation, the regime used in those patients with an increased incidence of skin cancer through the years has been combined azathioprine and prednisolone therapy. The results of this study suggest that neither of these two drugs is likely to be a direct promoter of skin cancer through the mechanism of reduced epidermal cell proliferation. When the two drugs were given to mice at approximately equivalent clinical dosages and at dosages that have previously been shown to be immunosuppressive (Kelly *et al.*, 1985), azathioprine had no effect on epidermal cell mitosis, while prednisolone had only a moderate depressive effect.

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# Eosinophilic pustular folliculitis. Studies on possible chemotactic factors involved in the formation of pustules

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## SUMMARY

Eosinophilic pustular folliculitis (EPF) is a dermatosis of unknown aetiology, characterized by repeated development of pruritic follicular papulopustules with a tendency to form an annular configuration on the face and other seborrhoeic areas, and by palmoplantar pustular lesions in one-fifth of the patients. Both types of lesions are infiltrated mainly by eosinophils with some polymorphonuclear leukocytes (PMN).

To elucidate the mechanisms underlying pustule formation, we studied the chemotactic activity for leukocytes of the skin surface lipids (SSL) obtained from seborrhoeic areas. No specific chemotactic activity was detectable in stored SSL from patients with EPF. However, fresh SSL collected from the seborrhoeic areas of normal adults contained chemotactic substances for eosinophils and PMN which were labile on storage in air. In stratum corneum extracts from palmoplantar lesions of patients with EPF we demonstrated the presence of a 13000 molecular weight chemoattractant factor for PMN, the activity of which was partially inhibited by antiserum against C5a, and a low molecular weight lipid-soluble chemotactic factor for eosinophils, the activity of which was also lost on storage in air. Our findings suggest that these chemotactic factors play a role in the production of the characteristic pustular lesions of EPF.

Eosinophilic pustular folliculitis (EPF) was first described by Ise and Ofuji (1965). Since then, more than 90 cases have been reported from Japan, in contrast to only 5 cases reported from Europe (Takematsu *et al.*, 1985). It is characterized by the repeated development of crops of pruritic follicular papulopustules with a tendency to form an annular configuration on the face and other seborrhoeic areas, and by palmoplantar pustular lesions that occur in one-fifth of the patients. Histologically, there is degeneration and disintegration of follicular walls resulting in spongiotic vesicle formation in the outer root sheath, associated with infiltration of eosinophils.

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The palmoplantar lesions show subcorneal pustules containing eosinophils and polymorphonuclear leukocytes (PMN). Blood eosinophilia is found in most cases.

Since no studies have been made on the nature of possible chemotactic substances or the pathological mechanisms underlying pustule formation, we carried out investigations in three patients with EPF.

## METHODS

### *Patients*

Details of the first two patients with EPF have been reported elsewhere (Takematsu *et al.*, 1985).

Patient 1 was a 29-year-old Japanese woman who had had grouped pruritic papulopustules on the left cheek and the extensor surface of the left upper arm for three months. The hair follicles and sebaceous glands were infiltrated mainly by eosinophils and by some polymorphonuclear leukocytes (Fig. 1).

Patient 2 was a 21-year-old Japanese man with a 6-year history of pruritic pustular lesions on the soles and palms. He also suffered from pruritic red follicular papulopustules on the face and extensor aspects of the upper and lower extremities.

Patient 3 was a 22-year-old Japanese woman with a 2-year history of periodic appearance of pruritic papulopustules on the face every few weeks. Systemic treatment with clindamycin (600 mg four times daily) or minocycline (200 mg twice daily) produced no significant improvement. A satisfactory clinical result was achieved by treatment with indomethacin (75 mg thrice daily). Using this treatment newly appearing lesions could be suppressed within a few days.

### *Extraction and storage of skin surface lipids*

Skin surface lipids (SSL) were obtained from the faces and upper arms of the patients and of two healthy adult male subjects, using the method of Puhvel and Sakamoto (1977). The subjects were asked to wash their face with soap in the evening. The following morning, cotton swabs moistened in hexane were used to wipe the face and upper arms. The SSL were extracted immediately from the swabs with hexane. The SSL obtained from the patients with EPF were stored frozen in air and were subsequently found to be chemotactically inert for PMNs and eosinophils. Therefore, only SSL freshly collected from the normal subjects were used for the chemotaxis assay.

To investigate the stability of SSL on storage, SSL from the normal subjects were pooled and stored at −70°C for 1–7 days either under nitrogen gas or in air. Immediately before testing they were dried under nitrogen gas, weighed and mixed with phosphate-buffered saline (PBS) by vigorous pipetting.

### *Extracts of stratum corneum*

Water soluble components were extracted from the crusty scales obtained from the pustular lesions on the palms and soles of patient 2 in the same way as described previously (Tagami & Ofuji, 1977). Extracts were also prepared from plantar stratum corneum of two patients with tylosis as a sample of orthokeratotic stratum corneum.

### *Preparation of zymosan-activated serum*

Fresh serum obtained form a healthy male subject was incubated with 1% zymosan (TCL, Tokyo, Japan) at 37°C for 1 h and then at 56°C for 30 min. After centrifugation at 3000 rev/min for 10 min, the supernatant was used as a positive control in eosinophil chemotaxis.

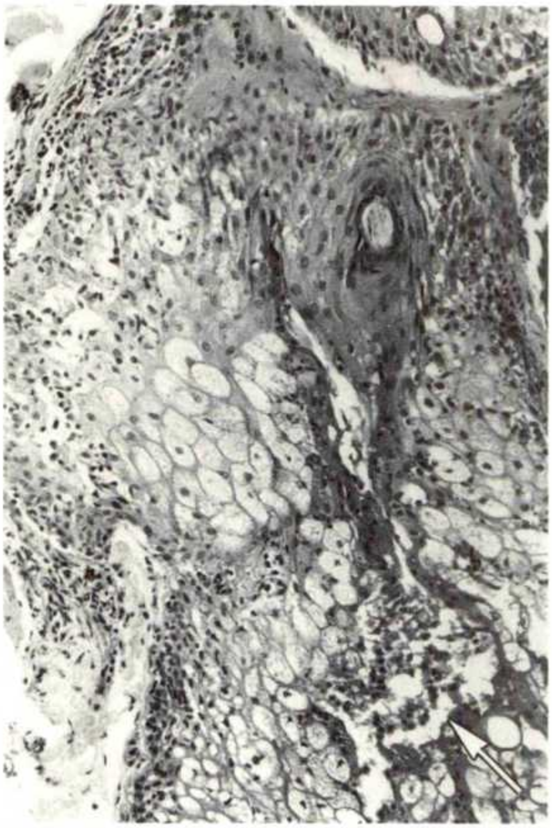

FIGURE 1. Histopathological features of the sebaceous follicle in EPF. Invasion of eosinophils is evident in the peripheral portion of the sebaceous gland with focal disintegration of its structure (arrow). (Original × 200).

### *Molecular sieve chromatography*

Fractions of stratum corneum extract for characterization of chemotactic factors were separated by gel filtration on Sephadex G-75 (2·6 × 50 cm) as reported previously (Tagami & Ofuji, 1977). Four millilitres of each sample was applied to the chromatography column and fractions of 5 ml were collected.

### *Assay of polymorphonuclear leukocyte chemotaxis*

All samples were assayed for chemotactic activity by a modification of the method described previously (Tagami & Ofuji, 1977). The upper compartment of a Blind Well Chamber (Labo Science, Tokyo, Japan) was separated from the lower compartment by a 3 μm Uni-Pore Polycarbonate Membrane (Bio-Rad Laboratories, Richmond, California, U.S.A.). At the end of a 45-min incubation period, the membranes were removed, stained with May–Giemsa, and the number of cells in a high power field were counted under × 400 magnification. For each chemotaxis assay, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (Sigma Chemical Company, St Louis, Missouri, U.S.A.) was used as a positive control.

We also tested the suppressive effect of rabbit antiserum to human C5a (a gift from Dr T.N.Hugli, La Jolla, California, U.S.A.) on chemotactic activity in stratum corneum extracts. Forty microlitres of the antiserum were incubated at 37°C for 30 min with 1 ml of the chemotactic fractions eluted near the cytochrome-c marker from column chromatography of the stratum corneum extracts, or with fractions of zymosan-activated serum. The treated fractions were then assayed in Boyden chemotaxis chambers and compared with fractions treated with non-immune rabbit serum or PBS.

### *Assay of eosinophil chemotaxis*

The chemotactic activity for eosinophils was measured by a modification of the method described by Czarnetzki, Kalveram and Dierksmeier (1979). Briefly, $2 \times 10^6$ guinea-pig peritoneal exudate cells, containing at least 40% eosinophils in a total of 0·2 ml were placed above an 8 μm Millipore filter (Millipore Corporation, Bedford, Massachusetts, U.S.A.) and the test substances below the filter, each in a total volume of 0·2 ml, in chemotaxis chambers. After incubation for 3 h at 37°C and 100% humidity, the filters were removed, stained with Mayer's haematoxylin and chromotrope 2R. Eosinophils that had migrated through the filter in 10 randomly selected high power fields were counted. In each chemotaxis assay, the activity of zymosan-activated serum was tested as a positive control.

### *Effect of boiling and ether extraction*

The effect of heating and of ether extraction on the chemotactic activity for eosinophils was studied in the fraction of stratum corneum extract from EPF which showed the maximum chemotactic activity for eosinophils (Fraction 20). An aliquot of the sample was boiled for 10 min. For ether extraction, 2 ml of Fraction 20 was extracted three times into two volumes of diethyl ether (Wako Pure Chemical Industries, Tokyo, Japan). After evaporation of the ether phase, the sample was reconstituted in 2 ml of PBS and aliquots were tested in the chemotaxis assay.

## RESULTS

### *Chemotactic activity of SSL for PMN and eosinophils*

All the samples of SSL from patients and normal subjects, which had been stored for more than one month at −70°C in air were found to be chemotactically inert. SSL freshly obtained from

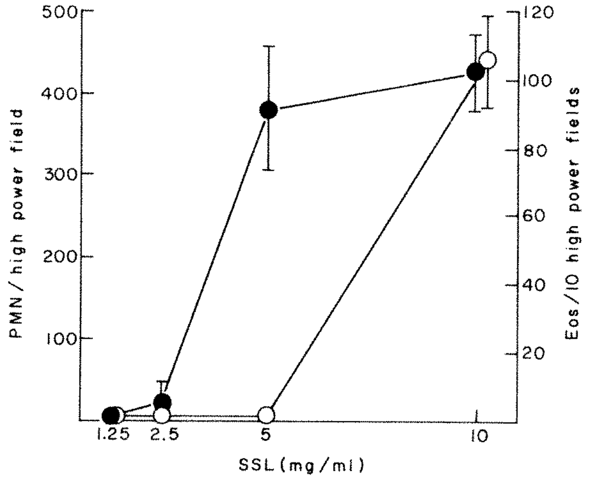

FIGURE 2. Chemotactic response of guinea-pig eosinophils (○) and guinea-pig PMN (●) to fresh SSL from normal subjects.

the normal subjects attracted PMN in a dose-dependent fashion (Fig. 2). They were also weakly chemotactic for eosinophils.

The chemotactic activity of SSL was lost after 24 h storage at −70°C in air, whereas SSL stored at −70°C under nitrogen retained their original activity after 7 days. Since the SSL collected from the patients had lost their chemotactic activity on storage no results of chemotaxis assays for SSL from EPS patients were obtained.

*Chemotactic activity of stratum corneum extract from palmoplantar lesions*

The extract prepared from normal plantar stratum corneum from the two patients with tylosis gave only low molecular weight PMN chemotactic fractions on Sephadex G-75 chromatography. The extract of plantar stratum corneum from patient 2 showed a multiphasic pattern of PMN chemotactic activity, with a definite peak in the fractions eluted near the cytochrome c

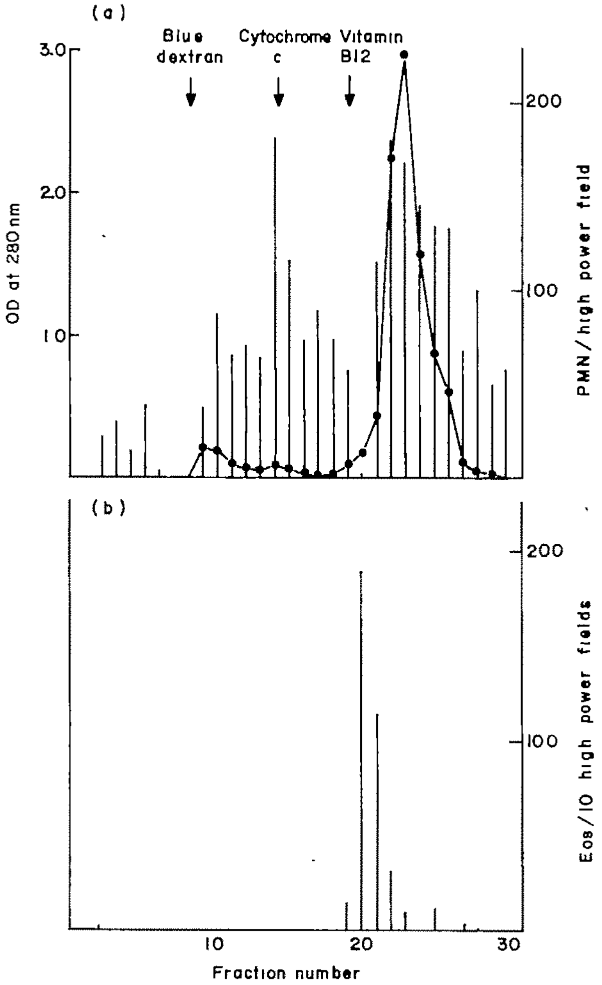

FIGURE 3. Chemotactic activity of Sephadex G-75 fractions of stratum corneum extract from EPF lesions (a) PMN chemotaxis; (b) eosinophil chemotaxis. Vertical bars represent chemotactic activity; points joined by a solid line represent OD at 280 nm.

TABLE 1. Inhibition of chemotactic activity in Sephadex G-75 fractions by rabbit antiserum to human C5a

| | PMN/high power field (mean ± s.d.) | |
|---|---|---|
| | Anti-human C5a | Normal rabbit serum |
| EPF, Fractions 13–15 | 194 ± 28 | 269 ± 22 |
| Zymosan-activated serum, Fractions 13–15 | 19 ± 6 | 155 ± 5 |

Negative control (PBS in place of chemoattractant) 6 ± 2 PMN/high power field.

TABLE 2. Effect of boiling and ether extraction on the eosinophil chemotactic activity of Sephadex G-75 Fraction 20

| Treatment | Eosinophils/5 HPF* (mean ± s.d.) |
|---|---|
| Untreated | 255 ± 34 |
| 100°C for 10 min | 264 ± 22 |
| Ether extraction | 264 ± 19 |

* High power field.

marker (molecular weight, 13000 daltons) (Fraction 14) (Fig. 3a). Treatment with rabbit anti-C5a serum produced only a partial reduction in this chemotactic activity, although the same treatment produced a marked inhibition of chemotactic activity in the fractions similarly prepared from zymosan-activated serum (Table 1).

The chemotactic assay for eosinophils showed the pesence of a single peak of chemotactic activity in the fraction near the vitamin B12 marker (Fraction 20; Fig. 3b). As shown in Table 2, this material was heat-stable and could be extracted with diethyl ether. Its chemotactic activity for eosinophils was stable after 24 h storage at −70°C in air, but was lost on storage at −70°C in air for 4 weeks.

## DISCUSSION

Our results show that fresh SSL from seborrhoeic areas of normal subjects, and lesional stratum corneum extracts from the palmoplantar pustules of EPF contain chemotactic factors for PMN and eosinophils. Sebaceous gland activity has been suspected as a cause of the accumulation of eosinophils in the hair follicles in EPF because the lesions are mostly confined to seborrhoeic areas and there is a history of acne vulgaris in many cases (Ofuji *et al.*, 1970). Our *in vitro* results indicated that the SSL from normal adults contained labile chemotactic factors for both PMN and eosinophils. However, we have no proof that these are derived from the sebaceous follicles. The lability of the chemotactic factors in air suggests that easily oxidizable, lipid-soluble substances in the SSL may be responsible. An arachidonic acid metabolite subclass, leukotriene $B_4$, is a possible candidate (Soter *et al.*, 1983, Grabbe *et al.*, 1984). On the other hand, on the basis of the remarkable improvement in Patient 3 on systemic treatment with the cyclooxygenase inhibitor indomethacin, it is possible that the cyclooxygenase-generated metabolite HHT

(12-L-hydroxy-5,8,10-heptadecatrienoic acid) may play an important role in the development of skin lesions of EPF, as suggested by Miyauchi *et al.* (1985).

Molecular sieve chromatography of stratum corneum extract from palmoplantar pustular lesions showed the presence of a chemotactic factor for PMN with a molecular weight near 13 000 daltons, and a low molecular weight chemotactic factor for eosinophils. The chemotactic factor for PMN, part of which appears to be a C5-cleavage product, is similar to that demonstrated in psoriasis and pustular psoriasis (Tagami & Ofuji, 1977), pustulosis palmaris et plantaris (Tagami & Ofuji, 1978) and subcorneal pustular dermatosis (Tagami *et al.*, 1983). Regarding the low molecular weight chemotactic factor for eosinophils, we could not carry out the necessary analysis to determine whether it was identical to that found in SSL because of the shortage of material. If these two factors are the same substance, they cannot originate from sebum since there are no pilosebaceous follicles in the palmoplantar skin.

From the consistent finding of blood eosinophilia in EPF, it may be postulated that this is a systemic disorder affecting blood and skin. We do not think that the eosinophil chemotactic factors demonstrable locally are also resonsible for blood eosinophilia. They probably play a role only in the accumulation of circulating eosinophils in the skin lesions. With regard to palmoplantar lesions, the results showed a striking similarity with those in pustulosis palmaris et plantaris. However, further investigation is required to elucidate the precise mechanisms underlying the cutaneous lesions of EPF as well as the nature of the chemotactic factors involved.

## ACKNOWLEDGMENTS

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# The effect of Goeckerman therapy on interferon in serum and suction blister fluid from patients with psoriasis

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## SUMMARY

An infectivity inhibition micromethod was used to detect interferon (IFN) in sera and suction blister fluids from 35 patients with untreated psoriasis vulgaris. IFN ($\geq 16$ units/ml) was detected in 56% of the sera (median 25 units/ml), in 77% of the suction blister fluids from lesional skin (median 35 units/ml) and 33% of the blister fluids from unaffected skin (median 10 units/ml). IFN levels were significantly higher in blister fluids from lesional skin than from unaffected skin ($P < 0.05$) indicating local IFN production. Results of characterization experiments indicated the presence of both acid stable and acid labile IFN-$\alpha$ as well as IFN-$\gamma$ in sera and blister fluids. After Goeckerman therapy, IFN was detected in 91% of the sera (median 89 units/ml), in 90% of the blister fluids from lesional skin (median 50·5 units/ml) and in 72% of the blister fluids from unaffected skin (median 26·5 units/ml). The IFN levels in sera were significantly higher than in blister fluids from both lesional skin ($P = 0.05$), and unaffected skin ($P = 0.001$). Furthermore, after Goeckerman therapy the IFN levels in blister fluids from unaffected skin and in sera were significantly higher than those in untreated patients ($P = 0.01$ and $P = 0.0001$) respectively. The results indicate that UVB radiation induces systemic IFN production.

Three main types of interferon (IFN) are found in humans (Stewart *et al.*, 1980). The type produced depends on the cell type and on the inducer. IFN-$\alpha$ is mainly produced by leukocytes in response to various stimuli, in particular viruses. IFN-$\beta$ is produced by fibroblasts (Stewart *et al.*, 1980). IFN-$\gamma$ or immune IFN is produced by lymphocytes, mainly T-lymphocytes, when these are stimulated by antigens (Green, *et al.*, 1970), mitogens (Wheelock, 1965), immune complexes or antilymphocytic antibody (Fujibayashi, Hooks & Notkins, 1975). It is possible

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Some of these data were presented in the 14th Annual Meeting of the European Society for Dermatological Research, Amsterdam, 1984 and at the 15th Annual Meeting of the Scandinavian Society for Immunology, Odense, 1984

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that IFN may contribute to the pathogenesis of several diseases, due to the wide range of its effects on the immune system and on cell growth. IFN has been detected in sera from patients with systemic lupus erythematosus (SLE), vasculitis, Sjøgren's disease (Hooks *et al.*, 1982; Preble *et al.*, 1982), and rheumatoid arthritis (Degre, Mellbye & Clarke-Jenssen, 1983). Recently, we have demonstrated IFN-$\alpha$ and IFN-$\gamma$ in suction blister fluids and sera from patients with psoriasis (Bjerke *et al.*, 1983). In the present paper, the results of extended observations on IFN activity in psoriasis are presented together with the effect of Goeckerman therapy on IFN levels. The data obtained may help to explain the beneficial effect of Goeckerman therapy.

## METHODS

### *Patients*

Thirty-five patients with psoriasis vulgaris (eight female and 27 male, average age 42·7 years, range 15–72 years) were studied. Topical treatment was stopped for at least one week prior to the study and none of the patients received artificial ultraviolet (UV) radiation therapy for one month before examination. None of the patients received systemic treatment. Twenty-two patients with psoriasis vulgaris (three female and 19 male, average age 44·3 years, range 15–72 years) were studied after they had received Goeckerman therapy (UVB radiation and 3% pyroleum lithantrasis in Unguentum Merck® 3–6 times weekly. Each patient received an average of 24 treatments (range 10–42). Eight of these patients (one female and seven male, average age 38·0 years, range 28–60) were examined both before and after 20 Goeckerman treatments.

### *UVB radiation*

A Waldmann UV 1000 treatment cabin (Waldmann Co., Willingen-Schwenningen, West Germany) equipped with 26 Sylvania UV6 fluorescent sunlamps were used as the UVB source. At 310 nm the Waldmann 1000 has an output 1·5–1·8 mW/cm$^2$ as stated by the manufacturer. The dose was gradually increased using suberythematous doses, starting with 50 m Joules/cm$^2$ and with 1200 m Joules/cm$^2$ as the maximum dose.

### *Blister fluids and sera*

Suction blisters were produced both in lesional and unaffected skin (at least 10 cm away from lesions) using the method described by Kiistala (1968). After introducing an electric vacuum pump it was unnecessary to treat the lesions with de-scaling agents. Before the cups were applied the coal tar ointment was removed by washing. The pressure within the cups was maintained constant at 175 mm Hg below atmospheric pressure. Serum samples and blister fluids were collected from the patients at the same time. In lesional skin there was often a considerable leakage of fluid through the fragile roof of the blisters. This fluid was pooled with the fluid obtained from within the blisters of each patient. The blister fluids and sera were stored at −70°C.

### *Interferon assay*

IFN activity was tested by an infectivity inhibition micromethod (Dahl & Degre, 1972), employing human embryonic lung fibroblast cells, prepared in our laboratory, in their fifth to fifteenth passage, and vesicular stomatitis virus (VSV), originally obtained from Dr E. Norrby, Stockholm, as the challenge virus. Two-fold dilutions of the samples were added to the cell cultures in triplicate, incubated overnight at 37°C and challenged with 10 infectious units of

VSV. A titration of VSV was included in each test. After three days incubation the virus caused a marked cytopathogenic effect on the cells. Interferon-treated cells were protected. The antiviral activity expressed in IFN units/ml was calculated as the reciprocal of the highest dilution preventing development of the cytopathogenic effect in 50% of the cells at the time when control cells were completely destroyed (Reed & Muench, 1938). This concentration was defined as containing one unit of IFN in our assay. One unit of the international standard IFN preparation (G-023-901-527) obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, Md, U.S.A., was equivalent to 1–1·3 units in our assay system. As some sera produced a nonspecific depression of virus plaque formation at low dilutions, only sera containing antiviral activity of 16 units/ml or higher were considered positive (Degre, Dahl & Vandvick, 1976; Hooks *et al.*, 1979). The same criterion was applied to the blister fluids.

To characterize the antiviral activity, representative samples were tested for activity on mouse L-929 cells, obtained from Dr H. Schellekens, Primate Centre, Rotterdam, Holland, and also tested for heat sensitivity. Heat sensitivity was examined by testing samples after incubation for 1 h at 56°C. The effect of low pH on the IFN activity was also tested. Samples of 0·2 ml of 13 sera and 10 blister fluids were adjusted to pH 2·0 by adding 1 M HCl. The samples were incubated at 4°C for 2 days, readjusted to pH 7·2 by adding 1 M NaOH and tested for IFN activity as described. A reduction of the IFN activity of 50% or more was considered significant. Treatment at pH 2 abolished the antiviral activity of the human IFN-$\gamma$ preparation obtained from Dr Marc De Ley, University of Louvain, Belgium, but did not affect the activity of human IFN-$\alpha$ obtained from Dr Kari Cantell, National Institute of Public Health, Helsinki, Finland.

Specific anti-human IFN-$\alpha$ antiserum was obtained from the Antiviral Programme of the National Institute of Allergy and Infectious Diseases, Bethesda, MD, U.S.A. One ml of this preparation (diluted 1:10 000), neutralized 10 units of human IFN-$\alpha$. In the neutralization assay the anti-human IFN-$\alpha$ was used at a dilution corresponding to twice the number of units of IFN in the sample. The diluted antiserum was incubated with an equal amount (0 1 ml) of undiluted serum or blister fluid for 1 h at room temperature and then overnight at 4°C. The mixture was then assayed for IFN activity as described above. Three serum samples and three samples of blister fluids were tested for antiviral activity in a bovine kidney cell line (MDBK) obtained from Dr C. Chany, Hospital St. Vincent de Paul, Paris, France. VSV was used as the challenge virus. Antiviral activity in the MDBK cells is specific for human IFN-$\alpha$. Human IFN-$\beta$ and IFN-$\gamma$ are inactive on these cells (Preble *et al.*, 1982).

### *Statistics*

The results are given as the median, as the variables are not normally distributed. The median levels were compared by the methods of Wilcoxon and Mann-Whitney. $P$ values $\leq 0{\cdot}05$ were considered to be significant.

## RESULTS

Before Goeckerman therapy, IFN was detected in blister fluids from lesional skin in 27 of 35 patients (77%, median 35 units/ml), and in blister fluids from unaffected skin in seven of 21 patients (33%, median 10 units/ml) (Fig. 1). The median IFN level in blister fluids from lesional skin was significantly higher than the median level in unaffected skin ($P < 0{\cdot}05$). IFN was detected in 19 of 34 of the patients' sera (56%, median 25 units/ml). The difference between the median IFN level in blister fluids from lesional skin and the corresponding sera was, however, not statistically significant ($P = 0$ 24).

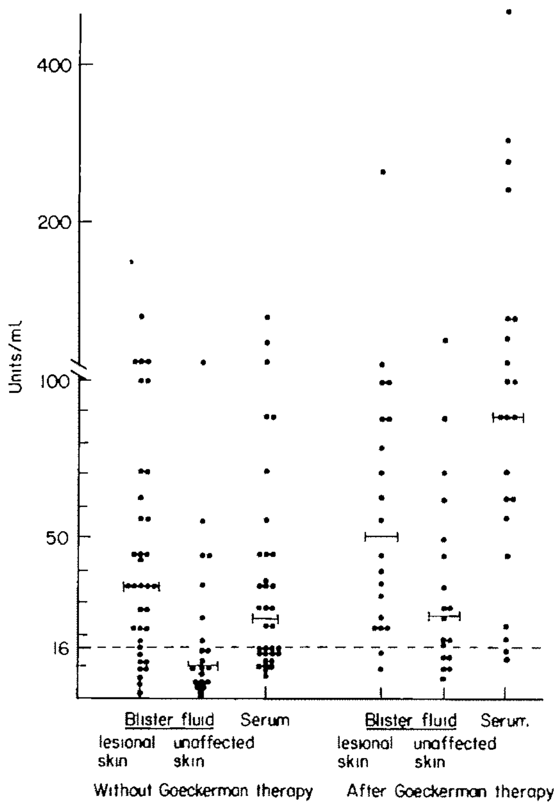

FIGURE 1. Interferon levels in blister fluids and sera from patients with psoriasis vulgaris without, and after, Goeckerman therapy. ⊢—⊣ = median.

After Goeckerman therapy the IFN levels were higher in blister fluids from both lesional skin and unaffected skin, as well as in sera (Fig. 1). IFN activity was found in blister fluids from lesional skin in 18 of 20 patients (90%, median 50·5 units/ml), in blister fluids from unaffected skin in 13 of 18 patients (72%, median 26·5 units/ml) and in 20 of 22 of the patients' sera (91%, median 89 units/ml). The median IFN levels both in blister fluids from unaffected skin and in sera were significantly higher in the patients receiving Goeckerman therapy than in the untreated patients ($P = 0{\cdot}01$ and $P = 0{\cdot}0001$ respectively). There was no statistically significant difference in the median levels of IFN in blister fluids from lesional skin before and after Goeckerman therapy, ($P = 0{\cdot}15$). The highest IFN levels were found in the treated patients. Furthermore, after Goeckerman therapy the IFN levels in sera were significantly higher than the IFN levels in blister fluids from unaffected skin ($P = 0{\cdot}001$) and from affected skin ($P = 0{\cdot}05$).

An enhancing effect on the IFN levels by Goeckerman therapy was also indicated by the results obtained in the 8 patients examined before and after Goeckerman therapy (Fig. 2). Before therapy, IFN was detected in blister fluids from lesional skin in six patients (75%, median 40 units/ml), in unaffected skin in two patients (25%, median 10·5 units/ml), and in sera from five patients (62·5%, median 28·5 units/ml). After therapy, IFN activity was found in blister fluids from lesional skin in seven of eight patients (87·5%, median 50·5 units/ml), in unaffected skin in five of seven patients (62·5%, median 23 units/ml), and in seven of eight sera

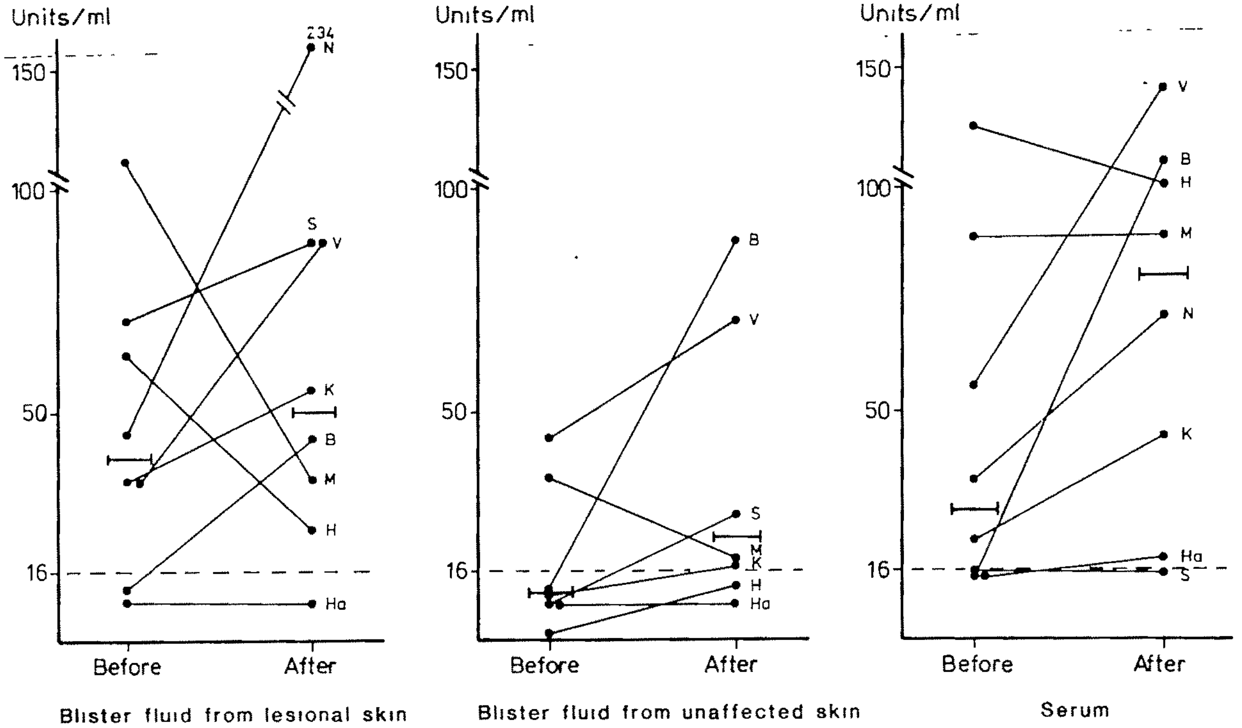

FIGURE 2. Interferon levels in blister fluids and sera from eight patients with psoriasis vulgaris before and after Goeckerman therapy. ├──┤ = median. (Letters are patient's' initials).

(87·5%, median 80 units/ml). The median IFN levels both in blister fluids and in sera were higher after Goeckerman therapy than the corresponding levels before treatment. However, the numbers were too small to detect a statistically significant difference. In three of the patients (M, H, Ha), either lower or unchanged IFN levels in blister fluids and sera were found after Goeckerman therapy (Fig. 2).

The antiviral activity in blister fluids and sera was characterized as IFN by its species specificity (no activity was obtained when tested in mouse L-929 cells), by its heat sensitivity and its lack of direct neutralization of the virus. Treatment at pH 2 reduced the antiviral activity in eight of 13 sera and in seven of 10 blister fluids, indicating the presence of IFN-γ (Table 1). Four sera and two blister fluids showed unchanged IFN activity indicating the presence of IFN-α and/or IFN-β. The presence of IFN-α was also evident from the fact that the IFN-α antiserum reduced the IFN activity in four of 10 sera and six of 11 blister fluids (Table 1). After treatment at pH 2, all three sera and two of the three blister fluids showed reduced IFN activity when tested on the MDBK cells, indicating the presence of both acid stable and acid labile IFN-α (Table 2).

TABLE 1 The effect of incubation at pH 2 and of anti IFN-α antibody on IFN activity in sera and blister fluids from patients with psoriasis

| | | No. of samples with IFN activity | | | |
|---|---|---|---|---|---|
| Treatment | Samples | reduced | unchanged | increased | Total |
| pH 2 | Sera | 8 | 4 | 1 | 13 |
| | Blister fluids | 7 | 2 | 1 | 10 |
| Anti-α IFN | Sera | 4 | 6 | 0 | 10 |
| | Blister fluids | 6 | 5 | 0 | 11 |

TABLE 2. IFN levels (units/ml) in sera and blister fluids before and after treatment at pH 2 when tested on bovine cells (MDBK)

| | Serum sample no. | | | Blister fluid sample no. | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| Before pH 2 | 112 | 224 | 89 | 22 | 35 | 45 |
| After pH 2 | 18 | 18 | 32 | 22 | 18 | 20 |

## DISCUSSION

The results obtained agree with, and also supplement, our previous observations that anti-viral activity characteristic of IFN can be detected in sera and blister fluids from patients with psoriasis (Bjerke *et al.*, 1983). Furthermore, the data corroborate our earlier assumption that IFN is produced locally in the psoriatic lesions, most probably by activated T-lymphocytes (Bjerke *et al.*, 1983). This is consistent with the finding that the median IFN level in blister fluids from the psoriatic lesions was significantly higher than in blister fluids from unaffected skin and higher than that in serum. The IFN levels both in sera and blister fluids in this study were generally higher than the IFN levels reported in our previous study (Bjerke *et al.*, 1983). We have no obvious explanation for this apparent disagreement. However, it may be partly explained by variability in the biological IFN assay. The small amount of IFN in blister fluids from unaffected skin probably comes from serum through passive diffusion. However, the possibility that IFN is also produced in unaffected skin cannot be excluded.

The results obtained in the characterization experiments indicate that the antiviral activity both in blister fluids and in sera is due to IFN-$\gamma$ and IFN-$\alpha$. Activated T-lymphocytes are probably the main producer of IFN-$\gamma$ (Valle *et al.*, 1975). The demonstration of IFN-$\gamma$ indicates that cellular immune reactions occur in psoriatic skin, and are possibly involved in the pathogenesis. In the present study we also demonstrated an acid labile type of IFN-$\alpha$ both in blister fluids and in sera. This is of particular interest since acid labile IFN-$\alpha$ has recently been demonstrated in serum from patients with autoimmune disorders, such as SLE, vasculitis, scleroderma and Sjøgren's disease (Hooks *et al.*, 1982; Preble *et al.*, 1982). Previously, IFN-$\gamma$ was demonstrated in sera from such patients. However, data reported by Cesario *et al.* (1983) indicate that the anti-viral activity is predominantly due to acid labile IFN-$\alpha$. The demonstration of IFN-$\gamma$ and acid labile IFN-$\alpha$ in both sera and blister fluids is therefore of interest in the consideration of psoriasis as a possible autoimmune disorder (Beutner, 1982; Krogh & Tønder, 1972). Acid labile IFN-$\alpha$ has also been found in sera from patients with acquired immunodeficiency syndrome (AIDS) (Eyster *et al.*, 1983) which probably is caused by a retrovirus (Gallo *et al.*, 1983), as well as in serum from patients with influenza (Green *et al.*, 1982). The cell producing acid labile IFN-$\alpha$ has not yet been identified. The presence of acid stable as well as acid labile IFN-$\alpha$ in sera and blister fluids may lend support to the hypothesis of a viral aetiology in psoriasis. Our recent demonstration of retrovirus-like particles in suction blister fluids from psoriatic lesions (Bjerke *et al.*, 1984) and the demonstration of similar particles in scales and urine from patients with psoriasis (Dalen *et al.*, 1983) further supports this hypothesis.

During Goeckerman therapy, increased levels of IFN were demonstrated both in sera and blister fluids. Since UVB radiation is the most effective part of the Goeckerman therapy (Belsito & Kechijian, 1982) the increased levels of IFN may be due to UVB radiation. Previously, it has been shown that UV radiation can induce IFN production in human cells *in vitro* (Lindner-Frimmel, 1974). Recently, Diezel, Waschke and Sonnichsen (1983) found that photochemical treatment with psoralen and UVA radiation enhanced IFN levels in sera from all the psoriatic patients they examined. The unchanged or decreased IFN levels in three of our patients remain an enigma. Taken together, the data suggest that both UVA and UVB radiation can enhance the IFN levels in patients with psoriasis. Recently, we have found that UVA and UVB radiation also increase serum IFN levels in healthy individuals (unpublished data).

After Goeckerman therapy, the highest IFN values were found in sera, indicating a systemic effect of the UVB radiation. The increased IFN levels in suction blister fluids from unaffected skin may be due to a passive diffusion from serum. However, local IFN production by keratinocytes mediated by UVB radiation cannot be excluded. In addition to diffusion from serum, an enhanced production of IFN by keratinocytes or mononuclear cells in the lesions may explain the increased levels of IFN in blister fluids from lesional skin.

The significance of IFN in psoriasis is at present unknown. Because of the multiple effects of IFN in general it might be expected that IFN could contribute to the pathogenesis of psoriasis. IFN or IFN precursors increase autoimmune disease activity in New Zealand mice (Engleman *et al.*, 1981). Furthermore, endogenous IFN is largely responsible for the manifestations of acute lymphocytic choriomeningitis virus disease in suckling mice (Riviere *et al.*, 1980). That

IFN may be a mediator of the disease activity in SLE has been considered (Ytterberg & Schnitzer, 1982). Accordingly, IFN may play a role in diseases where immunological mechanisms are involved in the pathogenesis. The effect of IFN on immune functions may either be enhancing or inhibitory, according to timing, concentration and the IFN type involved. It is therefore tempting to speculate whether the beneficial effects of Goeckerman therapy may be mediated by IFN. Since different types of IFN have different biological functions, further data on the effect of Goeckerman therapy on IFN types are needed.

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***Pharmacology and Treatment***

# Comparison of two occlusive bandages in the treatment of venous leg ulcers

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## SUMMARY

A randomized controlled study was performed in 34 patients with venous leg ulcers. No difference was found in the healing rate between a new hydrocolloid dressing plus compression, and a double layer bandage, consisting of an inner paste bandage (i.e. a stocking impregnated with zinc oxide paste) and an outer elastic bandage. The paramenters chosen for objective evaluation were stereophotogrammetric measurement of ulcer area and volume, and bacterial counts.

In an earlier controlled comparative study on different methods of topical treatment of venous leg ulcers, a double layer bandage (DLB) was considered the most suitable for routine treatment (Eriksson *et al.*, 1984a). Moreover, in a one-year trial in a medical care programme for venous leg ulcers this method was found to work well in practice (Eriksson, 1983). The results of a pilot study of ulcer treatment with a new hydrocolloid dressing (HCD) (Eklund *et al.*, 1983) encouraged us to compare the effect of this bandage with that of the DLB in venous leg ulcers, especially as there are now published reports of favourable results in treating leg ulcers with HCD (Friedman & Su, 1984).

## METHODS

The study was designed as a randomized open trial and included 34 outpatients with chronic venous leg ulcers, 25 women and 9 men with a mean age of 74·3 and 66·9 years, respectively. Informed consent was obtained before the patients entered the study. The general health of the patients was checked; special attention was given to the presence of congestive heart failure with oedema of the legs, which was treated if necessary. The clinical state of the venous peripheral circulation was assessed. Arterial circulation in the leg was estimated from segmental blood pressure measurements. Routine laboratory tests were performed including blood glucose and

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TABLE 1. Numbers of patients remaining in the trial at each examination

| | Weeks of treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | 0 | 2 | 4 | 6 | 8 | 10 | 12 |
| HCD | 17 | 17 | 16 | 12 | 9 | 7 | 6 |
| DLB | 17 | 17 | 17 | 13 | 10 | 8 | 7 |

serum zinc as well as epicutaneous patch tests. Results of treatment were assessed by stereophotogrammetric measurement of ulcer area and volume (Eriksson *et al.*, 1979), and bacteriological culture. These were measured before commencement of treatment and every 2 weeks thereafter until healing, or for 12 weeks if not healed. The numbers of patients who remained in the study at each examination are listed in Table 1. Specimens for bacteriological culture were taken with a charcoal-treated cotton swab after washing the ulcer with saline to remove secretions and debris. The swabs were transported in a modified Stuart medium for aerobic and anaerobic bacteriological examination.

### *Treatment*

The patients were randomly allocated for treatment with either a double layer bandage (DLB) or an adhesive hydrocolloid dressing (HCD). The DLB consists of an inner paste bandage [i.e. a stocking impregnated with zinc oxide paste (ACO-salvstrumpa®, ACO, Sweden)] and an outer elastic bandage (Tensoplast®, Smith & Nephew Ltd., Great Britain, or Porelast Acryl®, Lohmann, GmbH, Germany). The HDC (DuoDERM®, Squibb ConvaTec Inc., U.S.A.) is a compound of gelatin, pectin, carboxymethylcellulose sodium and polyisobutylene, plus an elastic bandage (Wero®, Weruli AG, Switzerland). At the start of treatment and at every change

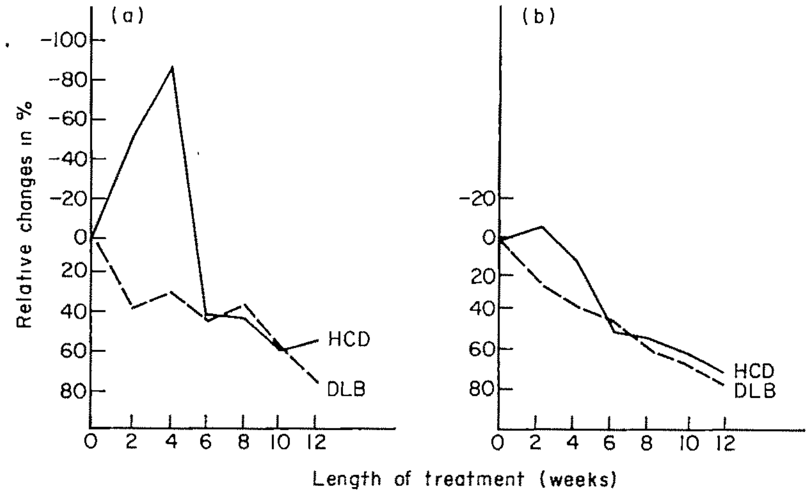

FIGURE 1. Relative changes in ulcer volume and area after 2, 4, 6, 8, 10 and 12 weeks of treatment with HCD + compression or DLB. Points are means. The figures on the ordinate indicate percentage changes relative to the value at the start of the study. The minus sign indicates an increase in (a) volume or (b) area.

of bandage the leg with the ulcer(s) was immersed for 15 min in a bath of tepid postassium permanganate (1 ml/l of a 3% solution), and crusts and debris were removed. The DLB was left in place for 1–2 weeks at a time. The HCD was changed once or twice a week, but the elastic bandage was removed at night and re-applied in the morning by the patient with or without help.

### *Statistical analysis*

Ulcer areas and volumes in the two groups were compared using a Student's *t*-test and healing of ulcers in relation to the bacterial flora was compared using a $\chi^2$ test.

## RESULTS

There was no significant difference in ulcer volumes between the two treatment groups at the start of treatment. Changes in the areas and volumes of the ulcers in the two groups after 2, 4, 6, 8, 10 and 12 weeks of treatment, expressed as a percentage of the initial measurements, are illustrated in Fig. 1. There was no statistically significant difference between the two treatment groups at any time point.

Ulcers in seven patients in the DLB group and nine in the HCD group healed during the 12-week period. Treatment was discontinued in five patients: one patient in each group dropped out. Two patients in the DLB group were regarded as clinical failures because of signs of infection in the skin surrounding the ulcer. In one patient in the HCD group the treatment was discontinued because of enlargement of the initial ulcer and development of a new ulcer.

*S. aureus* was the most common bacterial isolate. In general the 16 patients whose ulcers healed kept their initial flora until healing. The five patients with ulcers which initially yielded 'normal' skin flora or no growth did not differ in healing rate from the others. Gram-negative bacteria were found in ulcers that healed as well as in ones that did not.

Three patients, all in the HCD group, were diabetics. One healed within 3 weeks, in one the ulcer volume increased 150% and one was adjudged a treatment failure on clinical grounds because of enlargement of the initial ulcer and development of a new one. None of these three patients showed clinical signs of infection.

Six patients gave positive patch test reactions to elastic bandages, but none to HCD or to the inner layer of the DLB. In addition, nine patients gave positive reactions to wool alcohols, seven to neomycin, three to chinoform, three to parabens and six to rubber chemicals.

## DISCUSSION

With regard to bacterial growth, the present study confirms our earlier findings (Eriksson, Eklund & Kallings, 1984b). The patients seemed to keep their initial bacterial flora throughout the study whether they healed or not. The bacteria present in the ulcers were not found to be of clinical significance in the healing process. This study, however, was not designed to quantify the bacteria.

No statistically significant difference was found between the results of the two treatments. The large differences between the groups after 2 and 4 weeks of treatment were due to two extremely large ulcers, one later healing and one in a patient who dropped out after 4 weeks. The latter patient was clinically doing fairly well and was judged as slowly healing. To detect a minor difference, however, it would be necessary to enrol a much larger number of patients, which means that a study of this type would be very protracted. In view of the careful assessment of the patients' general health, evaluation of the peripheral circulation, laboratory and epicutaneous tests and bacteriological cultures, as well as an objective evaluation of the ulcer area and volume by stereophotogrammetry, the present study is probably a valid comparison, however. The two bandages both have advantages and disadvantages. The DLB may be changed less often than the HCD and is not disturbed between changes. Using HCD the patient can take a bath or shower with the dressing on, but must be able to re-apply the elastic bandage in the morning with or without help. Removal of the elastic bandage in the evening is easy.

As the incidence of contact allergy to topical remedies is high in patients with stasis ulcers and eczema, epicutaneous patch testing was performed in all patients. As expected several positive reactions were obtained. There were, however, no positive reactions to HCD or to the inner layer of DLB. Patients in the DLB group were, of course, treated with an outer elastic bandage which they tolerated.

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# Anti-oxidant action of metronidazole: a possible mechanism of action in rosacea

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## SUMMARY

To elucidate the mechanism of action of metronidazole in the treatment of rosacea, the effect of metronidazole on the generation of reactive oxygen species was examined both in neutrophil and xanthine-xanthine oxidase systems. Metronidazole had anti-oxidant action which was not exerted by the scavenging of reactive oxygen species, but by having an effect on neutrophil cell functions. Beneficial effects of metronidazole in the treatment of papulopustular rosacea can in part be attributable to this anti-inflammatory effect.

Oral metronidazole has been reported to be effective in the treatment of rosacea (Pye & Burton, 1976) and is equivalent in efficiency to oxytetracycline (Saihan & Burton, 1980). It has also been demonstrated that topical treatment with 1% metronidazole cream is as effective as systemic oxytetracycline therapy for rosacea (Nielsen, 1983a,b). The mechanism of the therapeutic response brought about by metronidazole is not established. We have therefore investigated the effect of metronidazole on the oxygen burst of neutrophils, because metronidazole is particularly effective against papulopustular rosacea (Pye & Burton, 1976) where inflammatory neutrophils are involved. It is therefore logical to establish whether metronidazole exerts its anti-inflammatory effect by affecting neutrophil cell functions.

## METHODS

Effects of metronidazole on the generation of reactive oxygen species (ROS) were investigated in neutrophils and in a xanthine-xanthine oxidase system. Metronidazole (Shionogi Pharmaceutical Co., Osaka, Japan) was dissolved in 50% dimethyl sulphoxide (DMSO) and 50% ethanol to give final concentrations of 1, 10 and 100 μg/ml. These concentrations were selected because the expected serum level of this drug after oral administration (500 mg/day) was known to be around 10 μg/ml. Each dose of the agent was added to the ROS generation system, and the inhibitory

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TABLE 1. Effects of metronidazole on the generation of reactive oxygen species

| Concentrations of metronidazole (μg/ml) | Reactive oxygen species generated by: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Zymosan-stimulated neutrophils* | | | | Xanthine-xanthine oxidase system* | | | |
| | $O_2^-$‡ | $H_2O_2$§ | OH·¶ | CL** | $O_2^-$ | $H_2O_2$ | OH· | CL |
| 0 | 1·56±0·18 | 2·14±0·14 | 8·61±0·29 | 85056±4365 | 0·71±0·08 | 0·98±0·12 | 1·29±0·21 | 21858±2157 |
| 1 | 1·59±0·20 | 1·99±0·22 | 8·88±0·22 | 81202±6028 | 0·72±0·07 | 1·00±0·17 | 1·36±0·17 | 18587±2013 |
| 10 | 1·60±0·17 | 1·76±0·13† | 6·10±0·13†† | 80938±5191 | 0·72±0·09 | 0·95±0·21 | 1·28±0·18 | 20038±1897 |
| 100 | 1·60±0·19 | 1·34±0·04†† | 5·71±0·30†† | 82706±5857 | 0·69±0·10 | 0·98±0·10 | 1·25±0·20 | 19392±2254 |

* Each value denotes mean ± s.d. of 3 assays.

‡ Superoxide (nmol/min/4 × $10^6$ neutrophils).

§ Hydrogen peroxide (nmol/min/2·5 × $10^6$ neutrophils).

¶ Hydroxyl radical (pmol/2 × $10^6$ neutrophils).

** Chemiluminescence (cpm/5 × $10^6$ neutrophils).

Comparison with control using Student's t-test † $P<0·1$ †† $P<0·05$.

effect on ROS generation was examined. The same volume of 50% DMSO and 50% ethanol was added to the control.

The methods used for assaying ROS have been detailed in previous reports (Miyachi & Niwa, 1982; Niwa, Sakane & Miyachi, 1984). In brief, neutrophils were isolated from peripheral blood by Ficoll–Hypaque gradient centrifugation and suspended in Krebs–Ringer phosphate buffer (KRP) and were preincubated at 37°C for 10 min with opsonized zymosan (Sigma). The formation of superoxide ($O_2^-$) was determined by measuring ferricytochrome c (Type III, Sigma, St Louis, Missouri, U.S.A.) reduction induced by $O_2^-$ produced from opsonized zymosan-stimulated neutrophils. Generation of hydrogen peroxide ($H_2O_2$) was measured by quantitating the decrease in fluorescence intensity of scopoletin (Sigma) due to its peroxidase-mediated oxidation by $H_2O_2$. Hydroxyl radical ($OH\cdot$) was assessed by the amount of ethylene gas formed from $\alpha$-ketomethiol butyric acid (Sigma) plus $OH\cdot$ generated by neutrophils. Chemiluminescence (CL) was measured in a scintillation spectrometer (Hitachi, Tokyo, Japan). In the xanthine-xanthine oxidase system, instead of adding neutrophils and opsonized zymosan, 0·1 ml of 13·5 mg hypoxanthine (Sigma) in 50 ml physiological saline plus 0·01 ml of 50 mM EDTA were diluted in 2 ml of KRP, and thereafter, 0·1 ml of 0·1 unit/ml dialysed xanthine oxidase (Sigma) was added to generate ROS.

## RESULTS

As shown in Table 1, metronidazole had an inhibitory effect on $H_2O_2$ generation by neutrophils in a dose-dependent manner at drug concentrations higher than 10 μg/ml. $OH\cdot$ production was significantly suppressed at drug concentrations above 10 μg/ml in zymosan-stimulated neutrophils. $O_2^-$ generation and CL were not affected by the drug. No significant effect of metronidazole was observed on any of ROS examined in the cell-free ROS generating system, i.e. xanthine-xanthine oxidase system.

## DISCUSSION

The present study demonstrates that therapeutic doses of metronidazole significantly reduced the levels of $H_2O_2$ and $OH\cdot$ which are considered to be potent oxidants capable of causing tissue injury at the site of inflammation. This effect was not observed in a cell-free xanthine-xanthine oxidase system, indicating that this anti-oxidant action is not exerted by the scavenging of ROS, but by having an effect on neutrophil cell functions. We have already shown that some anti-inflammatory agents are effective in various inflammatory dermatoses by decreasing ROS levels with resultant protection against auto-oxidative tissue damage (Miyachi & Niwa, 1982; Miyachi *et al.*, 1982). For example, the beneficial effects of dapsone are reasonably explained by its ROS quenching activity without affecting membrane phospholipid metabolism of neutrophils (Niwa & Miyachi, 1985). Although dapsone seems to be a more potent anti-oxidant, metronidazole seems to have a direct pharmacological effect on inflammatory cells, a different mechanism of action from dapsone.

Although it has been suggested that the effect of metronidazole is due to its anti-parasitic activity on *Demodex folliculorum* (Kurkcuoglu & Atakan, 1984), we suggest on the basis of our findings that metronidazole exerts its anti-inflammatory effects by anti-oxidant action. Beneficial effects of metronidazole in the treatment of rheumatoid diseases (Bingham, 1985) and psoriasis (Ely, 1985) could in part be ascribed to a similar mechanism. In this context, anti-malarial drugs which we have recently found to have direct effects on cell functions with subsequent anti-oxidant action (Miyachi *et al.*, in press) may be of value in rosacea.

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# Short contact anthralin in the treatment of psoriasis: a study of different contact times

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## SUMMARY

Eighteen patients with chronic plaque psoriasis were investigated in two groups to determine the optimal contact time for the application of 2% anthralin, within the range immediate removal to removal at 20 min. It was found that clinical efficacy, assessed by times to plaque clearing, and side effects of treatment, i.e. erythema and staining, were independent of anthralin contact time. There seemed to be a relationship between erythema induction and skin type.

The use of short contact anthralin (SCA) applications in the treatment of psoriasis has the advantage over the traditional 24-h regimens of convenience and increased patient acceptability. This is especially important for out-patients. The effectiveness of short contact treatments with relatively high anthralin concentrations has been established by several authors using different contact times, different concentrations and different bases. Runne & Kuntze (1982) used 1–3% anthralin with 5% salicyclic acid in yellow soft paraffin for contact times of 10–20 min ('minutes therapy') and in left-right comparisons they showed slightly accelerated clearing compared with a 24-h, lower concentration regimen. Marsden *et al.* (1983) showed that concentrations of anthralin in excess of 2% in emulsifying ointment with 0·5% salicylic acid, when left on for 30 min, while effective, induced significant erythema of non-lesional skin in a high proportion of patients, although these higher concentrations could generally be tolerated if their introduction was gradual. It was also apparent in this study that, although 30- or 60-min regimens were as effective at plaque clearing as the Ingram regimen, erythema occurred significantly more often with SCA. Statham, Ryatt & Rowell (1984) have reported that clearance with SCA in a soft paraffin base takes longer than with the Ingram regime unless the application is left on for 2 h. These authors used the same base as Runne & Kuntze but, unlike them, they used similar anthralin concentrations for the two regimens. They also found an increased irritancy with SCA.

The purpose of the present study was to explore the efficacy and side effects of 2% anthralin in an emulsifying ointment base for 'very short contact times' within the range immediate removal

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to removal after 20 min. It was further hoped to establish a protocol for its continued use even when erythema occurred.

## METHODS

### *Subjects*

These were 18 patients with chronic plaque psoriasis admitted for in-patient treatment. They were selected for the study if they had symmetrical lesions suitable for quarter and half body comparisons and were willing to take part. Patients were excluded on the grounds of past anthralin intolerance or intercurrent medical illness. The extent of the psoriasis was estimated according to the rule of nines, and skin was typed according to the history of burning and tanning:

Type I—always burn, never tan;
Type II—always burn, sometimes tan;
Type III—sometimes burn, always tan.

### *Materials and Methods*

Freshly prepared 2% anthralin with 0·5% salicylic acid in emulsifying ointment was applied to all lesional areas (scalp, face, neck and flexures excepted). The person applying it concentrated on the lesions but did not carefully confine it to them as with the Ingram regime. Removal (i.e.

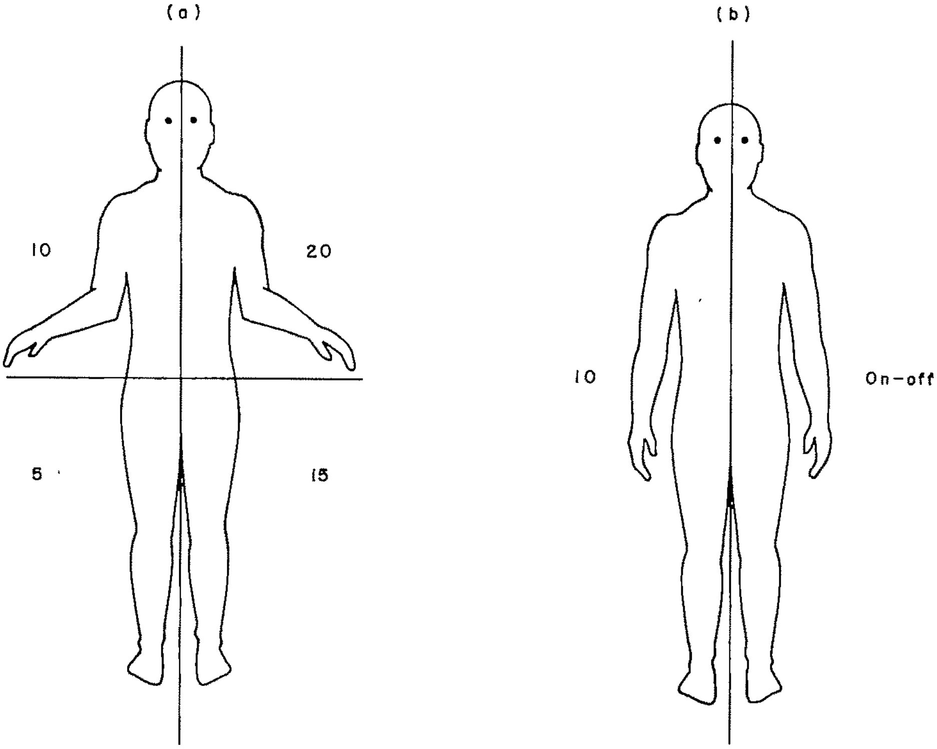

FIGURE 1. Contact times at different sites (see text). (a) Group 1: quarter comparisons ($n = 10$). (b) Group 2: left–right comparisons ($n = 8$).

removal of surplus) at the designated times was with Teepol detergent or Simple soap. Skin was subsequently dabbed dry with a cotton towel, and emulsifying ointment was applied and left on if the skin was uncomfortably dry.

Patients were divided into two groups. Group 1 (age range 12–65 years, extent of psoriasis 18–55%) was composed of ten patients (two of skin type I, four of skin type II and four of skin type III). Group 2 (age range 19–75 years, extent of psoriasis 12–48%) was composed of eight patients (two of skin type I, four of skin type II and two of skin type III).

Patients in Group 1 were treated in a quarters comparison of anthralin contact times, the times for the different quarters being 5, 10, 15 and 20 min. Those in Group 2 were treated in a left–right comparison with anthralin contact times of 10 min and immediate removal (in practice a contact time of 30 s to 3 min). Figure 1 illustrates the areas and duration of treatment. Patients were assessed on a daily basis with reference to:

(i) Days to clinical clearing of plaques of psoriasis; less than 1% residual surface area involved being acceptable as the end point;
(ii) para-lesional erythema on a 0–4 scale according to severity; and
(iii) staining on a 0–4 scale according to severity.

When detectable erythema occurred together with subjective burning, SCA was withheld and only emulsifying ointment applied until the skin had settled, at which time anthralin was re-introduced at the same concentration (2%). Patients who were clearing slowly by the start of the third week were subsequently treated with increasing anthralin concentrations of up to 6% (two patients in group 1 and four patients in group 2).

## RESULTS

### *Response*

Both treatment groups showed similar clearance times. There was no detectable difference between any quarters or halves (Table 1). In two patients, both in group 1, leg plaques cleared more slowly than upper body plaques. Two patients, one from each group, failed to clear completely, one because of an unwillingness to persist with anthralin because of uncomfortable plaque erythema and the other because of anthralin resistant plaques.

### *Erythema*

The degree of erythema experienced in both groups was similar though slightly greater in group 2 in which there was a similar distribution of the different skin types and shorter contact times.

TABLE 1. Days to clear plaque psoriasis on different comparisons

| | Anthralin contact time (min) | Mean days to clear ± s.d. |
|---|---|---|
| Group 1 (10 patients) | 5 | 22·89 ± 6·88 |
| | 10 | 21 88 ± 6·75 |
| | 15 | 22·89 ± 6·88 |
| | 20 | 21·88 ± 6·75 |
| Group 2 (8 patients) | 'on/off' | 21 71 ± 5 34 |
| | 10 | 21·71 ± 5·34 |

TABLE 2. Erythema of paralesional skin in different comparisons

Group 1 (Quarter comparisons)—10 patients

| | Duration (days) | | | | Daily score* | | | |
|---|---|---|---|---|---|---|---|---|
| | Contact time (min) | | | | Contact time (min) | | | |
| | 5 | 10 | 15 | 20 | 5 | 10 | 15 | 20 |
| Means | 1·2 | 1·3 | 1·2 | 1·6 | 0·6 | 0·6 | 0·6 | 0·6 |
| ±s.d. | ±1·7 | ±1·8 | ±1·7 | ±1·8 | ±0·8 | ±0·8 | ±0·8 | ±0·8 |

Group 2 (left–right comparisons)—8 patients

| | Duration (days) | | Daily score* | |
|---|---|---|---|---|
| | Contact time (min) | | Contact time (min) | |
| | on/off | 10 | on/off | 10 |
| Means | 1·4 | 1·9 | 0·7 | 0·8 |
| ±s.d. | ±1·9 | ±1·9 | ±0·8 | ±0·9 |

$$* \text{ Daily erythema score} = \frac{\text{summed daily severity scores (0–4 each day).}}{\text{total days duration of erythema}}$$

TABLE 3. Paralesional erythema according to skin type

| | |
|---|---|
| Skin Type I ($n = 4$) | (4 patients developed erythema) |
| Mean duration | 3·25 ± 1·5 s.d. days |
| Mean daily score | 1·74 ± 0·33 s.d. |
| Skin Type II ($n = 8$) | (3 patients developed erythema) |
| Mean duration | 1·25 ± 1·75 s.d. days |
| Mean daily score | 0·55 ± 0·77 s.d. |
| Skin Type III ($n = 6$) | (1 patient developed erythema) |
| Mean duration | 0·33 ± 0·82 s.d. days |
| Mean daily score | 0·17 ± 0·41 s.d. |

In neither group was there any appreciable difference between quarters or halves (Table 2). It was noted incidentally that flexural burning occurred in some patients, presumably due to spread of anthralin during the removal process. All four patients with type I skin, three out of eight with type II skin and one out of six with type III skin developed erythema. Though total numbers were small (Table 3), erythema scores were significantly greater in skin type I and II than in skin type III ($P < 0{\cdot}05$, Wilcoxon sum rank test).

### *Staining*

Staining was similar in all areas and did not correlate with contact time or skin type. By completion of treatment, scores ranged between 1 and 4. In many cases staining was very marked and showed both a striking 'halo' effect and extended well away from the treated plaque. This latter observation was probably due to the 'smear' method of application.

## DISCUSSION

Daily application of 2% anthralin and 0·5% salicylic acid in emulsifying ointment was effective in clearing plaque psoriasis in approximately 22 ± 6 s.d. days when in contact with skin for up to 20 min. The regimen was well tolerated from the start and the erythema seen in 50% of our patients could generally be controlled by replacing anthralin for up to 4 days with emulsifying ointment. Two per cent anthralin could then be reintroduced without further burning, with only one exception.

The time of contact prior to removal of surplus anthralin (i.e. the short contact time) did not influence either efficacy or side effects within the range of contact periods ( < 20 min). Even for the shorter treatment group, efficacy, burning and staining were the same. This suggests that removal was incomplete, or that anthralin rapidly bound to keratin and penetrated normal as well as psoriatic skin.

Our findings are at variance with those of Runne & Kuntze (1982) when shorter contact times conferred less erythema. We found an apparent correlation between skin type and anthralin induced para-lesional erythema. These findings are in keeping with those of Maurice & Greaves (1983) using a different method, but different from those reported by Lawrence *et al.* (1984).

An advantage of short contact anthralin in an emulsifying base is the convenient and easy method of application and removal which is in contrast to the difficulty experienced with the stiff base of the conventional dithranol Ingram regimen. Skin staining was, however, marked and staining of clothes and linen also occurred if removal of anthralin from all skin was not complete.

Our study suggests that patients treating their psoriasis at home with short contact regimens can remove anthralin almost immediately after application. Patients should be warned about staining of the bath. To prevent permanent damage the bath must be cleaned immediately after use (Pratt, 1984).

## ACKNOWLEDGMENT

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## *Case Reports*

# Necrobiotic xanthogranuloma: a report of four cases

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## SUMMARY

Four patients are described who demonstrate the range of clinical and pathological features seen in necrobiotic xanthogranuloma with paraproteinaemia (NXG). Each patient had the typical periorbital yellow plaques with numerous well demarcated yellow indurated plaques on the trunk. All four patients had evidence of paraproteinaemia. The histopathology showed diagnostic features of intense necrobiosis with xanthomatization, including touton giant cells and the bizarre angulated giant cells of NXG. Previously, the patients had been diagnosed as atypical necrobiosis lipoidica, and the distinction between NXG and other necrobiotic conditions is discussed.

Necrobiotic xanthogranuloma with paraproteinaemia (NXG) was delineated as a separate entity, distinct from necrobiosis lipoidica, by Kossard & Winkelmann (1980) who described eight patients with unusual lesions of the head, neck and trunk. These patients presented with sharply demarcated indurated plaques and nodules that were often bright yellow in colour and which had a predilection to occur on the head and neck and more especially the periorbital region. Crusting and recurrent or intractable ulceration was a common complication. Biopsies of the plaque revealed a distinctive histological pattern that combined pictures of severe necrobiotic changes with xanthogranuloma formation. An associated dysproteinaemia or evidence of myeloproliferative disorder was found in each case. Later Robertson & Winkelmann (1984) studied 16 cases and highlighted the ophthalmic complications, particularly scleritis, episcleritis, conjunctival plaques and sometimes keratitis and loss of sight. Most recently Finan & Winkelmann (1984) summarized 22 cases, emphasizing the distinctive pathology and response of lesions to oral alkylating agents.

Such cases are clinically and histologically distinctive, and several cases reported as unusual variants of necrobiosis lipoidica may retrospectively be recognized as necrobiotic xanthogranu-

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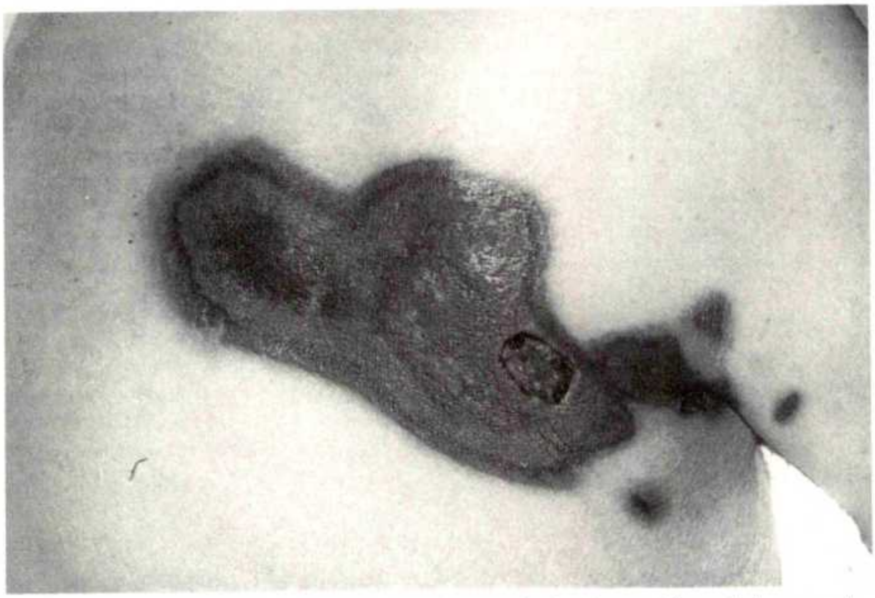

FIGURE 1. Case 1. An early lesion comprising a large haemorrhagic plaque with a well demarcated yellow edge and extensive areas of ulceration.

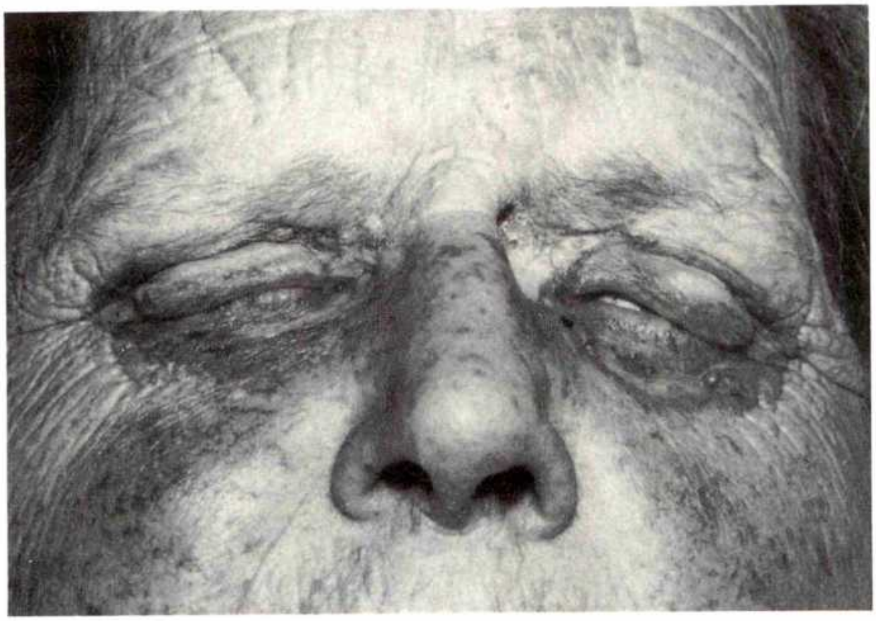

FIGURE 2. Case 1. Indurated yellow plaques present around both eyes. Recurrent ulceration has caused some scarring around both palpebral fissures.

loma (Mehregan & Pinkus, 1961; Navaratnam & Hodgson, 1973; Metz & Metz, 1977; Bowers, 1975; Mackey, 1975). Indeed, two cases were described as atypical necrobiosis lipoidica by Muller & Winkelmann (1966) that later would certainly have been classified as NXG.

This paper discusses four cases of NXG identified at St John's Hospital for Diseases of the Skin. Some of the cases had previously been classified as atypical necrobiosis lipoidica in the histological files.

## CASE 1

A frail 78-year-old lady presented originally with splenomegaly of unknown cause. She remained well, but 2 years later she developed multiple cutaneous lesions on the upper trunk and around the eyes. Six well demarcated plaques were present on the trunk ranging in size from a few centimetres to over 25 cm diameter. The plaques had vivid yellow, well demarcated edges and some showed black/brown haemorrhagic centres. Several of the plaques showed large areas of ulceration (Fig. 1). One lesion showed central atrophy with telangiectasia. The lesions around the eyes were indurated nodules and plaques, most prominent on the inferior portion of the orbit (Fig. 2). There was some scarring and superficial ulceration adjacent to the inner canthus. The palpebral fissure was reduced in size and the patient had xerophthalmia. There were no lesions on acral sites and none in the typical distribution of necrobiosis lipoidica on the shins. On general examination no lymphadenopathy and only slight splenomegaly was detectable.

Investigations revealed mild anaemia (11·9 g/dl) and a reduced total WCC ($3{\cdot}6 \times 10^{12}$/l) with a normal ESR. Numerous earlier plasma protein studies had been normal but recently a faint IgG monoclonal band with reduction of total globulins was found. Bence-Jones protein examinations of concentrated urine have been repeatedly negative. Urea and electrolytes, liver function, serum lipids, glucose tolerance and complement were normal, and cryoglobulins were not detected.

Several bone marrow examinations had shown a normal cellular marrow with a normal erythromyeloid ratio. However, one examination showed an increased proportion of lymphocytes bearing only one light chain type.

The patient's cutaneous disease has been partially controlled by prednisolone 30 mg once daily, but none of the lesions has cleared completely and new lesions have continued to arise. Treatment with chlorambucil 20 mg/day for 2 days every 3–4 weeks caused exacerbation of the lesions as did a similar regime of cyclophosphamide.

## CASE 2

A 64-year-old lady presented in 1974. She had a 3-year history of purplish patches developing on both cheeks with some central ulceration. Gradually, the cutaneous lesions progressed and she developed numerous plaques around the eyes, and on the face, neck and upper anterior chest. The lesions were centrally pigmented with a distinct yellow xanthomatous edge. In areas where lesions had subsided there was atrophy with telangiectasia.

The condition was originally diagnosed as sarcoidosis and she had previously been diagnosed as having sarcoid of the cervical lymph nodes in 1968. However, the condition had not responded to prednisolone (30 mg/day). There was a family history of diabetes. On examination the patient was clinically anaemic with hepatomegaly but no splenomegaly or lymphadenopathy.

Investigations confirmed the anaemia (Hb 6·4 g/dl) and showed a reduced WCC of $3{\cdot}2 \times 10^{12}$/l, with an ESR of 127 mm/h. Further investigations revealed iron deficiency and immunoelectrophoresis of plasma proteins showed a monoclonal band in the gamma globulin region. Bone marrow examination and skeletal survey were normal. Glucose tolerance test, serum lipids and liver function tests were also normal and cryogloblins were not found.

Extensive barium X-ray studies showed no cause for blood loss and the haemoglobin responded to transfusion and oral iron therapy.

### CASE 3

A 55-year-old lady presented with a chronic history of yellow, well-defined, infiltrated plaques in the left infraorbital area, on the breasts and in an appendicectomy scar. The patient also had splenomegaly. This patient had been reported originally as having sclerosing lipogranuloma, ?necrobiosis lipoidica by Bowers (1975) and later was designated as a case of atypical multicentric reticulohistiocytosis with paraproteinaemia (Rendall *et al.*, 1977).

The lesions enlarged over the following years with inflammation, ulceration and sinus formation. Subsequently, the areas became largely inactive with atrophy and telangiectasia.

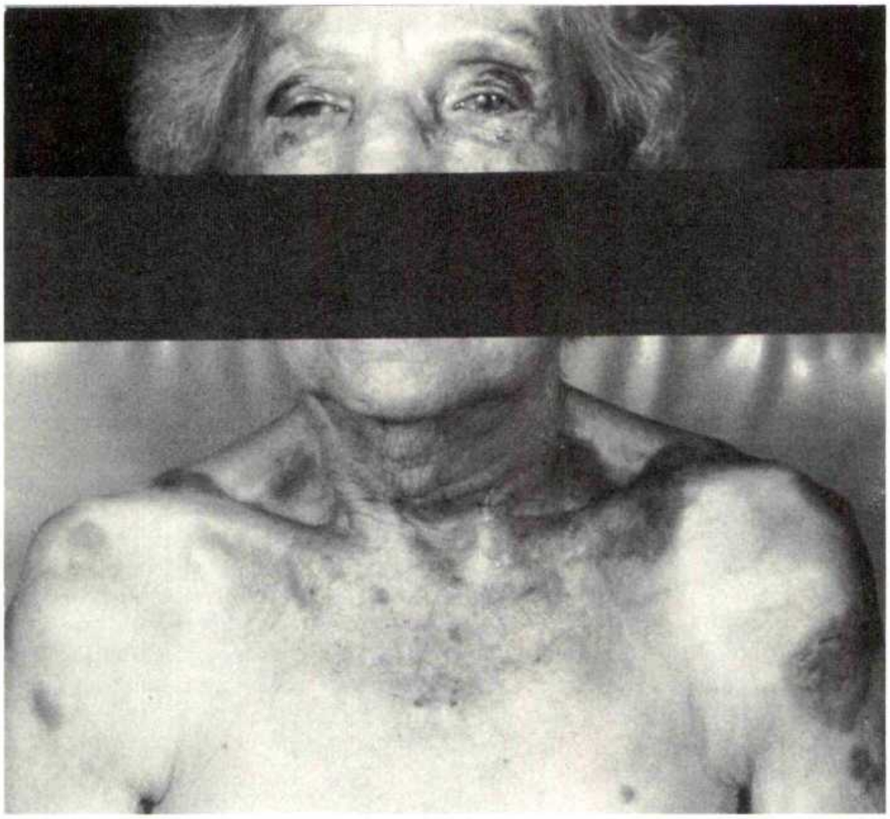

FIGURE 3. Case 4. Late lesions showing numerous extensive atrophic plaques of necrobiosis with periorbital accentuation.

Recently, she had an episode of haematemesis and was found to have oesophageal varices, probably secondary to primary biliary cirrhosis.

When reported by Rendall *et al.* (1977) the patient had a raised ESR (41 mm/h) with normal haematological indices and an IgG paraprotein. Liver function tests, serum lipids, glucose tolerance test and skeletal survey were normal.

Subsequently, the plasma paraprotein has persisted and the glucose tolerance test has become abnormal. In addition, the patient has developed a raised bilirubin and alkaline phosphatase (AP = 27 kAU/dl, normal < 11), positive ANA binding of low titre with normal DNA binding, positive mitochondrial antibodies and positive smooth muscle antibodies. Liver biopsy showed histology consistent with primary biliary cirrhosis.

## CASE 4

An 85-year-old lady was kindly referred by Dr S.Bor and has been presented at the Royal Society of Medicine as a case of atypical necrobiosis lipoidica.

She presented with weakness and weight loss associated with the eruption of painful and extremely tender nodules on her upper arms, left side of her neck and both shoulders, in addition to yellow-red plaques around the orbits. She had suffered from exophthalmic goitre when she was young, and in her 60's she had developed bilateral cateracts causing her to be registered blind 6 years before developing NXG. At presentation she had numerous, yellow, well demarcated plaques with areas of atrophy and ulceration on the upper body and around the eyes. No lymphadenopathy or organomegaly was detected. Prednisolone therapy produced improvement of many of the lesions and now at 89 years of age she has numerous well demarcated, but not ulcerated, atrophic yellow/white telangiectatic patches in similar areas to her original lesions (Fig. 3).

Haematological investigations showed mild anaemia 11·5 g/dl with an elevated WCC $10{\cdot}3 \times 10^{12}/\mathrm{l}$. These have progressed to 6·8 g/dl and a WCC of $24 \times 10^{12}/\mathrm{l}$. A small gamma band was present on immunoelectrophoresis which was subsequently shown to be an IgG paraprotein. In addition, tiny amounts of kappa light chain were found in the urine.

Bone marrow examination and skeletal survey were normal. Glucose tolerance testing showed a mildly diabetic curve and the patient had autoantibodies against thyroid and mitochondria. Other autoantibodies were negative. Serum lipids, liver function tests, urea and electrolytes, and complement were normal.

## HISTOPATHOLOGY

All four patients had the characteristic histopathology of NXG, although there was some variability in the features from case to case. All showed large areas of severe necrobiosis alternating with bands of granulomatous infiltration that extended throughout the dermis into the subcutis (Fig. 4). The necrobiosis was usually sharply marginated from the surrounding granuloma and was particularly severe, showing a complete obliteration of normal collagen structure. The appearance was that of structureless eosinophilic debris resembling caseous material. The granuloma comprised a mixture of lymphocytes, epithelioid cells, foamy histiocytes and some touton giant cells. Adjacent to the areas of necrobiosis, numerous very large and bizarrely angulated multinucleate giant cells could be seen, their nuclei irregularly grouped together within the abundant eosinophilic cytoplasm of the cells (Fig. 5). These distinctive cells were present in all biopsies and appear to represent a characteristic feature of this condition.

In two cases cholesterol clefting could be seen in the areas of necrobiosis and this was particularly prominent in Case 1 (Fig. 4). The proportion of foamy histiocytes and touton giant

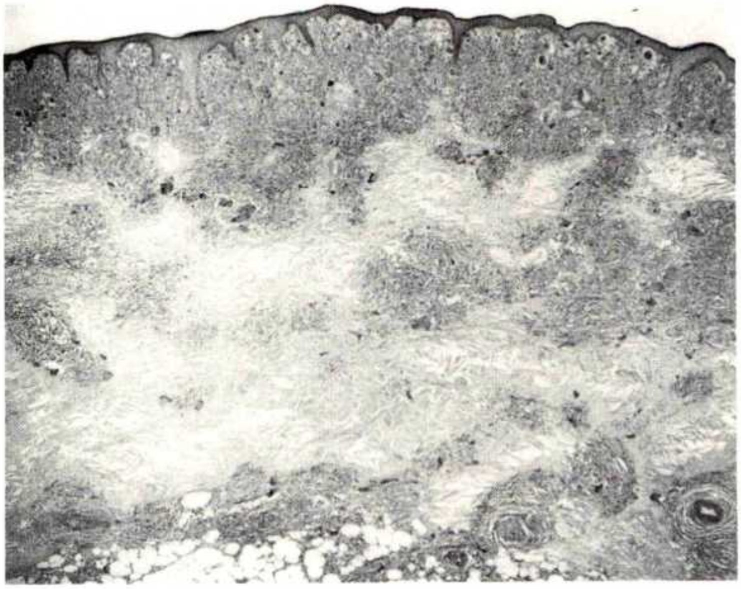

FIGURE 4. Case 1. Sections show extensive necrobiotic areas of collagen surrounded by tracts of xanthomatous and granulomatous infiltrate. Marked cholesterol clefting is present within the necrobiotic zones. Haematoxylin and eosin, (original × 28).

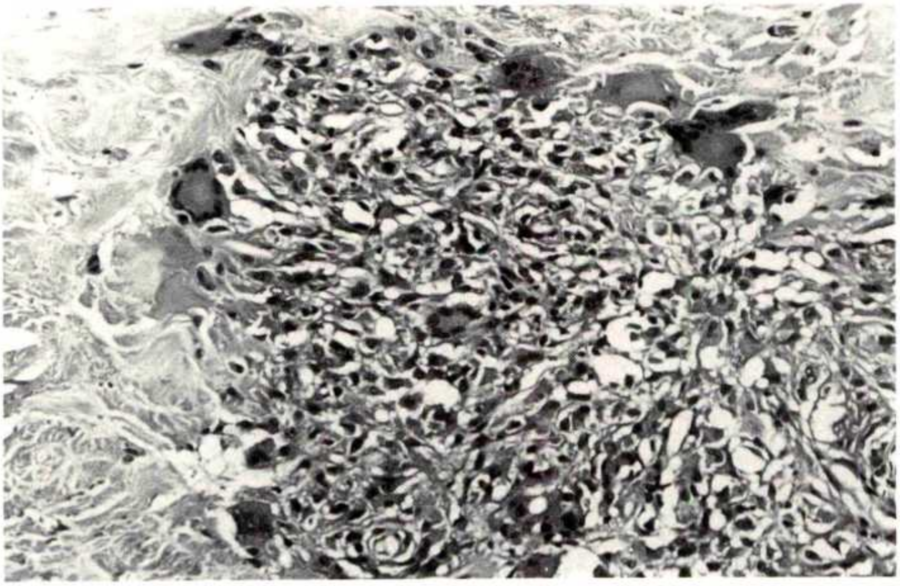

FIGURE 5. Case 1. Numerous bizarre giant cells with an angulated outline. Their nuclei are collected in irregular clumps within the eosinophilic cytoplasm. Haematoxylin and eosin, (original × 280).

cells in the infiltrate varied from case to case but was unusually prominent in the upper dermis in Case 1 which showed extensive infiltration by confluent areas of these cells (Fig. 6).

In Case 2, at the edges of the biopsy, there were areas of necrobiosis that were less severe and bore some resemblance to ordinary necrobiosis or granuloma annulare.

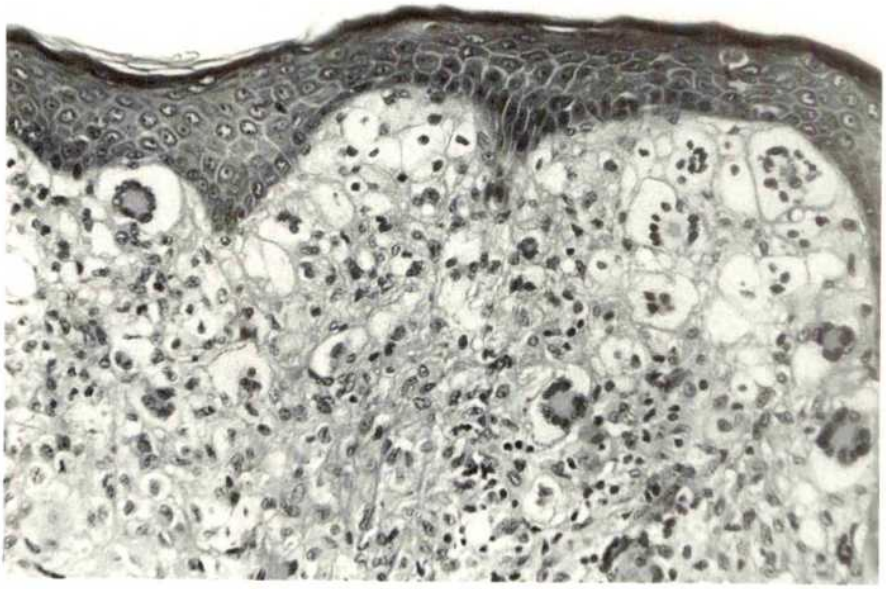

FIGURE 6. Case 1. Extensive xanthomatization is present in the papillary dermis and numerous touton giant cells can be clearly seen. Haematoxylin and eosin, (original × 280).

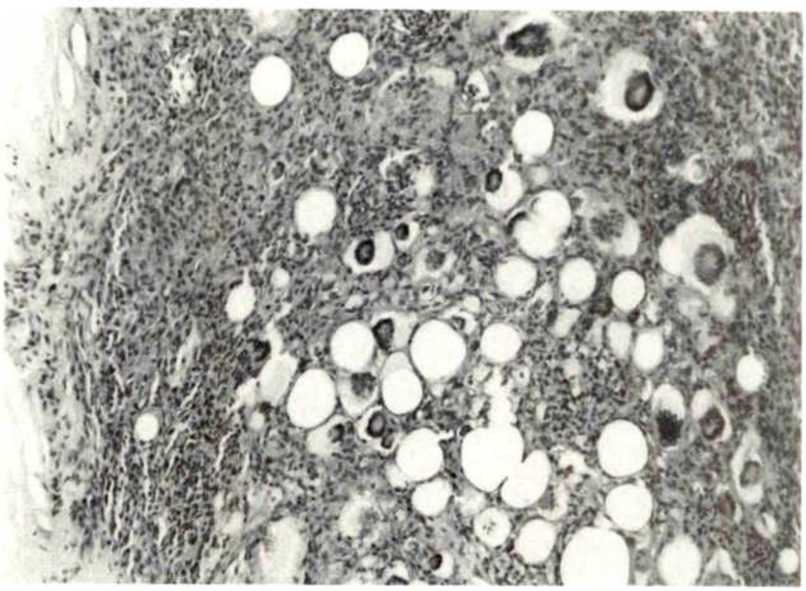

FIGURE 7. Case 3. Xanthogranulomatous change can be seen within the fat, including numerous touton giant cells. Haematoxylin and eosin, (original × 280).

Case 3 had a more prominent lymphocytic infiltrate which was diffuse and focal. There was marked involvement of the subcutis and in some areas aggregates of lymphoid cells were seen. Extensive xanthogranulomatous change with numerous touton giant cells was present in the subcutis (Fig. 7).

Case 4 had the typical histology of NXG except that the major component was a diffuse histiocytic granulomatous infiltrate with less prominent necrobiosis. Numerous atypical giant cells were found around the necrobiosis with a significant number of touton giant cells.

## DISCUSSION

These four cases were originally designated by our department as unusual variants of necrobiosis lipoidica. However, all four may be retrospectively designated necrobiotic xanthogranuloma, as described by Kossard & Winkelmann (1980). The condition is very rare and the four cases described represent less than 1% of the cases of necrobiosis lipoidica reported on histologically at St John's Hospital.

The case histories of our patients confirm the association of the characteristic pathology with paraproteinaemia and demonstrate the progressive chronic nature of the disorder. The clinical appearances of the patients were strikingly similar, as were the histopathological changes, enabling the condition to be readily recognised. Periorbital involvement was observed in all our patients and severe and recurrent ulceration of some of the lesions was a complication. In one patient NXG developed in a pre-existing scar. This has previously been reported in extensive necrobiosis lipoidica (Sahl, 1978), but not in necrobiotic xanthogranuloma.

All of the patients showed a partial response to prednisolone therapy, but in no case did the lesions disappear completely. One patient has received cytotoxic therapy with no response. However, this was given in high dosages not in the daily low dosage recommended by Robertson & Winkelmann (1984). Although all four patients had evidence of dysproteinaemia, none have developed myelomatosis as yet.

However, two of our patients had splenomegaly and in one patient (Case 1) this antedated the cutaneous changes. Although lymphadenopathy is unusual, splenomegaly or hepatomegaly was reported in five of Robertson & Winkelmann's (1984) series.

A similar range of laboratory abnormalities was seen in our patients as has been previously reported. Leukopenia and anaemia were common, but one patient has developed an elevated WCC. Three of our four patients showed an elevated ESR.

An abnormal paraprotein band was present in all our cases, but repeated examinations were required to demonstrate its presence in two cases. There was no evidence of myeloma on bone marrow examination or skeletal survey but in Case 1 one of a series of marrow examinations showed a monoclonal population of lymphocytes. The exact nature of this abnormality is still undetermined.

No patient had hyperlipidaemia and none were clinically diabetic. However one patient (Case 3) eventually developed an abnormal glucose tolerance test.

Interestingly, none of our patients showed evidence of cryoglobulins or reduced levels of complement components as seen in some patients previously reported. Two of our patients had evidence of autoimmune disease (primary biliary cirrhosis and Graves' disease, respectively) and both had persistent autoantibodies. These associations were not found in the larger series described by Robertson & Winkelmann (1984), but these authors noted positive rheumatoid factor in two cases.

The histological appearances showed the spectrum of change described by Kossard &

Winkelmann (1980), and were easily identified by the bizarre angulated giant cells in the granuloma, associated with extensive necrobiosis and varying degrees of xanthomatization. The severity of the changes distinguished the cases from typical necrobiosis lipoidica. In particular, the degree of xanthomatization with touton cell formation and the numerous giant cells with a bizarre angulated appearance suggest NXG rather than necrobiosis lipoidica.

The histology of our cases differs greatly from the condition described by Dowling & Wilson Jones (1967), and Wilson Jones (1971) as atypical necrobiosis of the face and scalp. The changes in the latter condition are never so destructive and histologically the necrobiotic changes are slight. Although giant cells are observed they are not as bizarre and angulated as those seen in NXG.

It was of interest that Case 3 showed lymphoid aggregates as described by Kossard & Winkelmann (1980) in two of their cases, and originally described by Winkelmann & Welbourne (1969). Our Case 3 has been reported previously as an example of atypical multicentric reticulohistiocytosis (MRH). We consider that this patient better fits the diagnosis of NXG. Extensive necrobiosis would be unusual for MRH, and the morphology of the giant cells is typical of NXG and not that seen in MRH. In the latter condition the giant cells are distinct, rounded or ovoid with several small well-defined discrete nuclei with a 'cats-eye' appearance. In addition this patient had extensive panniculitis with touton giant cell formation in the fat ('Touton cell panniculitis'), a finding only reported in NXG.

In a large series of necrobiotic xanthogranuloma cases, Robertson & Winkelmann (1984) reported 16 patients. Fifteen had ophthalmic complications ranging from periorbital ulceration, direct involvement of the orbit or deposits in the episcleral tissues causing recurrent symptoms of scleritis and episcleritis. The authors found that surgical treatment of these lesions was often followed by recurrence, and they suggested low dose chemotherapy as the treatment of choice. Corticosteroid therapy with melphelan or chlorambucil produced remission in the paraproteinaemia and of the skin lesions in a substantial number of their cases.

Our four cases demonstrate the clinical and pathological appearances of NXG and show the associated paraproteinaemia. However, the pathogenesis of NXG and its relationship to necrobiosis lipoidica remains unknown.

## ACKNOWLEDGMENTS

The authors are grateful to Dr Roberts who referred Case 1 and to Dr J.M.Boss and Dr S.Bor who kindly allowed us to report their patients (Cases 3 and 4).

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# Discoid lupus erythematosus-like lesions in an autosomal form of chronic granulomatous disease

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## SUMMARY

Chronic granulomatous disease (CGD) is characterized by a bactericidal defect involving the oxidative metabolism of polymorphonuclear leukocytes (PML) and is most often transmitted as an X-linked trait. The cutaneous features of this disorder include infections and lupus-like rashes. These have been described in female carriers as well as in males with the disease.

Two cases of siblings presenting an autosomal form of CGD syndrome, with lupus-like cutaneous manifestations, are reported here.

## CASE REPORTS

### *Case 1*

A 16-year-old girl had recurrent eczematoid and impetiginous lesions of the face and flexures for 10 years. Aged 5 years, she developed lymphadenopathy after BCG vaccination. CGD was diagnosed at the age of 11 on the basis of bactericidal anomalies and the absence of oxidative burst in polymorphonuclear neutrophils (PMN) (Table 1). In 1983, she was hospitalized for multiple hepatic abscesses due to *Staphylococcus aureus*. These were treated by surgical drainage and systemic antibiotics (amoxicillin 3 g/day for 1 month). In the past year, infiltrated, bluish-red papules have appeared on the sides of her fingers (Fig. 1).

Histological examination of one of the lesions showed moderate epidermal atrophy with discrete thickening at the dermoepidermal junction. Indirect immunofluorescence study of the lysed skin showed no immunoglobulin deposits. There were no circulating antinuclear antibodies, and anti-DNA antibodies and complement fractions (C2, C3, C4, CH50) were normal.

### *Case 2*

The 23-year-old elder brother of Case 1, has no history of infections. Despite this, he has presented biological anomalies of PMN killer function similar to case 1 (Table 1). Over the past 4 years he has developed rosacea-like facial lesions on his forehead and cheeks (Fig. 2) and has had bluish-red infiltrated plaques on the sides of his fingers. The condition of these lesions is improved by hydroxychloroquine, but deteriorates when therapy is discontinued. Histological examination of the face (Fig. 2) shows vacuolar degeneration of an atrophic epidermis and

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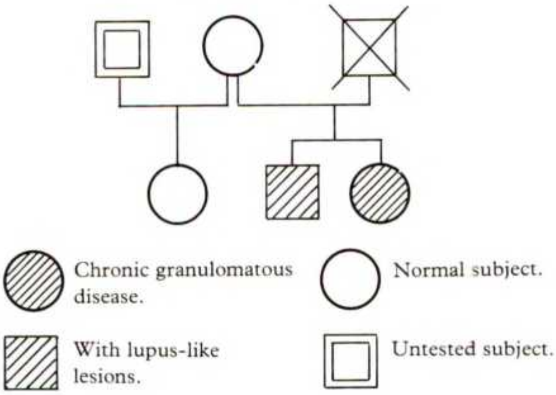
TABLE 1. After stimulation by zymosan, PMNLs from the patients failed to reduce nitroblue tetrazolium (NBT), to increase $O_2$ consumption or to generate $H_2O_2$ production

| | Normal | Case 1 | Case 2 | Mother | Half-sister |
|---|---|---|---|---|---|
| NBT reduction (% PMN) | 60 | 1 | 1 | 48 | 46 |
| Spontaneous $O_2$ consumption (nat/min/$10^6$ PMN) | 1·6 ± 0·7 | 0·3 | 0·3 | 2 | 4 |
| Stimulated $O_2$ consumption (nat/min/$10^6$ PMN) | 1·6 ± 2·5 | 0·4 | 1·4 | 15·5 | 10·4 |
| $H_2O_2$ production (nat/min/$10^6$ PMN) | 6·2 ± 1·1 | undeterminable | 0·7 | 7·2 | 4·6 |

TABLE 2. Neither the mother nor the half-sister of the two patients presented an anomaly in PMN killer function



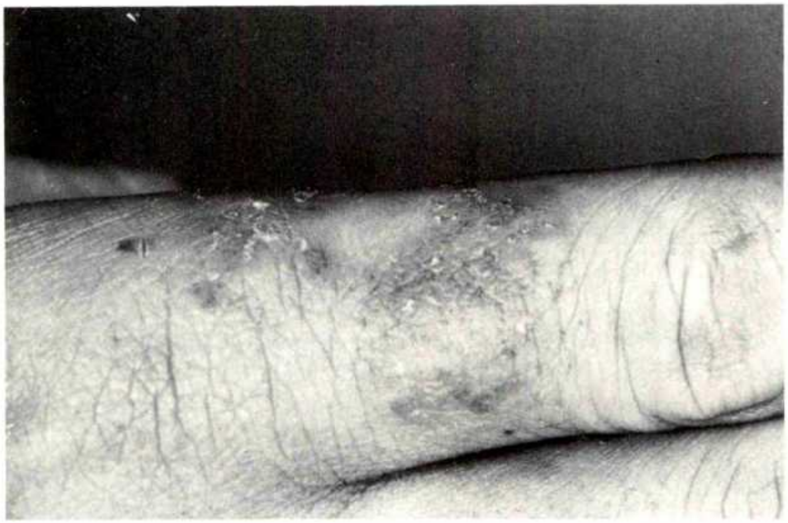


FIGURE 1. Infiltrated bluish-red papules on the sides of fingers. (Case 1).

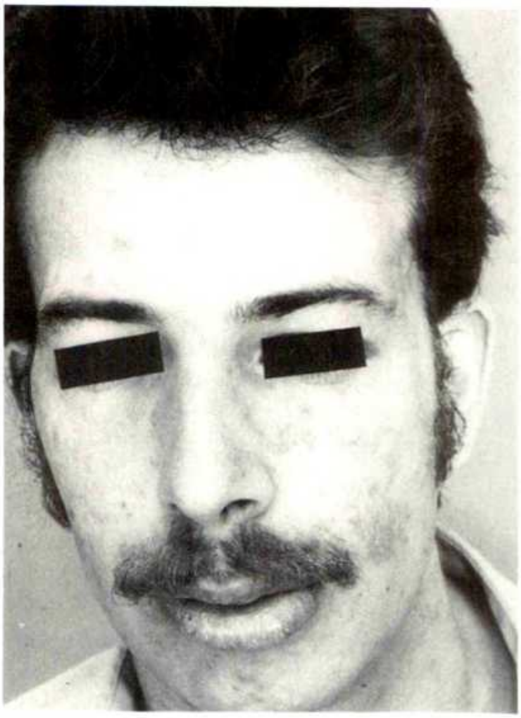


FIGURE 2. Rosacea-like eruption on the face. (Case 2).

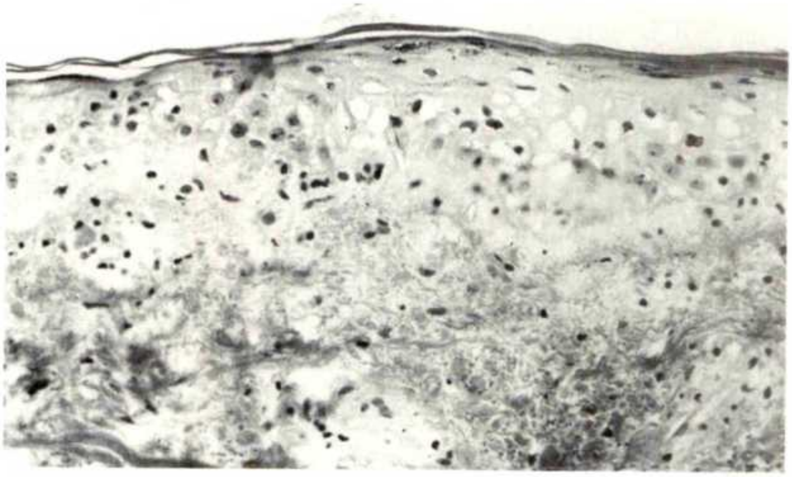


FIGURE 3. Vacuolar degeneration and thickening of the basement membrane.

thickening of the basement membrane. The dermis contains a predominantly perivascular and perifollicular lymphocytic infiltrate. Indirect immunofluorescence is negative.

Functional studies were performed on the patients' mother and half-sister. The father is deceased (Table 2).

## DISCUSSION

CGD is defined as a congenital hereditary syndrome characterized by severe and multiple infections associated with functional PMN abnormalities linked to defects in oxidative burst during bacterial killing (Quie, White & Good, 1967). Hereditary transmission is most often sex-linked as evidenced in a grouping of 168 cases (Johnston & Newman, 1977) involving 114 boys (sex-ratio 6:1). In mothers of affected children, intermediate rates of reduction by nitroblue tetrazolium (NBT) have been found which illustrate the Lyon hypothesis (Windhorst & Good, 1967). In effect, two types of PMN population have been detected, one abnormal and the other reducing NBT normally, (Ochs & Igo, 1973).

Cutaneous lesions have been described in 'female carriers' and mothers of affected children (Schaller, 1972). These are bluish-red, infiltrated papular lesions of the face and hands suggestive of discoid lupus erythematous lesions (Nelson, Dahl & Goltz, 1977). Mucous membrane involvement is common as aphthous-like ulcerations.

The histology of infiltrated lesions has shown a dermal perivascular and perifollicular lymphocytic infiltration with hyperkeratosis and acanthosis of the papillary layer. Cutaneous immunofluorescence studies have not revealed any lupus-band immunoglobulin deposits (Brandrup *et al.*, 1981).

In the two cases reported here, the brother and sister presented with similar clinical and biological anomalies. However, at the time of writing, the boy has presented no serious infectious complications. The lupus-like cutaneous manifestations in both patients are similar to those described during X-linked CGD (Stalder, 1982).

The disease in brother and sister can only be explained by assuming an autosomal form which is probably recessive. This form of transmission has been reported for girls (Chandra, Cope & Soothill, 1969), and Azimi has indicated the presence of CGD in three sisters (Azimi, Bodenbender & Hintz, 1968).

To our knowledge, lupus-like cutaneous manifestations have not been reported before as part of the autosomal form of CGD, even in carriers. Though there is no known relation between lupus-like lesions and CGD, the possibility of such lesions in child carriers of the autosomal recessive type should be remembered in familial detection of CGD. The diagnosis of familial discoid lupus erythematosus should alert the physician to investigate the family for CGD.

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# Haematological disturbances and immune mechanisms in toxic epidermal necrolysis

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## CASE REPORT

A 2-year-old girl presented with painful, widespread erythema with evidence of epidermal shedding 2 days after starting amoxycillin and paracetamol to treat a throat infection. By the following day the epidermal shedding had spread and she was hospitalized 2 days after the onset of symptoms. The diagnosis of toxic epidermal necrolysis (TEN) was confirmed histopathologically from a skin peel that indicated dermo-epidermal cleavage and eosinophilic necrosis of epidermal cells. The patient was immediately placed in the intensive care unit. Therapeutic measures included attentive nursing, maintenance of fluid and electrolyte balance and prevention of infections. No corticosteroid therapy nor preventive antibiotic therapy were initiated. Her condition remained stable until the 8th day of hospitalization at which time it began to deteriorate rapidly. Broad spectrum antibiotic therapy (gentamicin 5 mg/kg/day, chloramphenicol 100 mg/kg/day, rifampicin 15 mg/kg/day) was started. Platelet and red blood cell transfusion was performed because of pancytopenia. Despite these measures, the patient died the next day. The results of the 8-day blood culture showed *Enterobacter cloacae* and *Escherichia coli* septicaemia. All cultures previously had been negative even at the time of maximum fever.

The patient had developed a progressively worsening aregenerative normocytic anaemia. The RBC count fell from 4476000 to 3180000/mm$^3$; Hb from 12·2 to 8·7 g/dl; MCHC 32·5%; MCV 83·3 and sternal puncture revealed erythroblastopenia. Even more striking, however, was the change in the white blood cell count (Fig. 1). At the time of admission, blood examinations showed a moderate leukopenia with neutropenia (WBC count of 2900/mm$^3$ with 30% neutrophils) and a predominance of 'band' forms. This condition worsened and by the 7th day of hospitalization, the day before the patient's condition deteriorated, the count had fallen to 1100 WBC/mm$^3$ with 40% neutrophils. Thrombocytopenia was also detected at this time (2900 platelets/mm$^3$). Sternal puncture revealed a bone marrow rich in immature granulocytes (promyelocytes, myelocytes and metamyelocytes) and deficient in polymorphonuclear neutrophils. On the 8th day, the white blood cell count had fallen to 700/mm$^3$ with 5% neutrophils and a predominance of band cells. There was no signs of disseminated intravascular coagulation (DIC) as prothrombin test time (PTT) and fibrinogen were normal.

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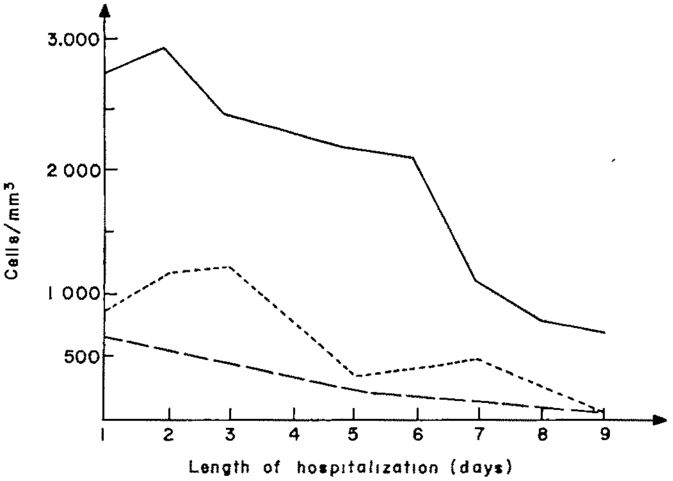

FIGURE 1. The rates of evolution of circulating white blood cells (——), neutrophils (· ·) and band cells (– – – –) during the period of observation.

## DISCUSSION

The mechanisms responsible for the epidermal necrosis in this condition remain controversial. To date, most hypotheses focus on the etiological role of immune mechanims.

There is little evidence for a role of humoral immunity, based mainly on infrequent detection by direct immunofluorescence of immunoglobin and complement deposits in the intercellular space of the basal cell layer (Stein *et al.*, 1972).

The importance of cell mediated immunity may be greater based on the clinical, histological and biological common features between TEN and graft versus host reaction (GVHR), the latter considered by some to be cell-mediated.

TABLE 1. TEN-related haematological disorders

| References | Number of cases | Anaemia | Neutropenia | Lymphopenia | Thrombopenia |
|---|---|---|---|---|---|
| Jarkowski *et al.* (1962) | 1 | — | 1 | — | — |
| Mitroshina *et al.* (1976)<br>Juldashev *et al.* (1978)<br>[quoted in Westly *et al.* (1984)] | 3 | ? | 3 | ? | ? |
| Haynes (1978) | 1 | 1 | 1 | — | — |
| Finlay *et al.* (1982) | 1 | ? | 1 | ? | ? |
| Ortiz *et al.* (1982) | 1 | 1 | 1 | 1 | 1 |
| Halebian *et al.* (1983) | 18 | ? | Unspecified. Leukopenia in at least five cases | 'Common' | ? |
| Bombal *et al.* (1983) | 26 | 25 | 3 | 24/25 | 2/8 |
| Westly *et al.* (1984) | 10 | — | 10 | ? | — |

A TEN-like eruption has been observed in patients who develop an acute graft versus host reaction (GVHR) either following a bone marrow transplant (Peck, Herzig & Elias, 1972) or after blood transfusion (McCarthy, Raimer & Jaratt, 1978). Furthermore, the syndrome has been experimentally reproduced in hamsters by inducing a GVHR with injection of parental lymphoid cells (Billingham & Streinlein, 1968). In GVHR, the occasional observation of satellite cell necrosis (binding of a lymphocyte to a necrotic keratinocyte; De Dobbeleer, Ledoux-Corbusier & Achten, 1975) suggests a direct cytotoxicity mechanism, while the cytotoxicity of sera from GVHR patients and laboratory animals for epidermal cell suspensions (Peck *et al.*, 1972) suggests an indirect mechanism, in which activated or derepressed lymphocytes produce an epidermotoxic lymphokine responsible for the basal cell layer necrosis.

In drug-induced TEN, the near absence of a dermal infiltrate, called dermal silence by Achten & Ledoux-Corbusier (1970), argues rather for an indirect cytotoxic process.

The same mechanisms may be responsible for the haematological disturbances in TEN patients. The frequency and significance of these disturbances have been stressed in recent studies (Bombal *et al.*, 1983; Westly & Wechsler, 1984) (Table 1).

A normocytic anaemia characterized by a progressive drop in haemoglobin accompanied by a moderate reduction in serum iron seems to be a common finding in TEN patients, as is indicated by Bombal's study (1983) where it was reported in 25 out of 26 cases. The peculiarity of this anaemia lies in its aregenerative character with medullary erythroblastopenia and blood reticulocytopenia. It does not appear to be related to the inflammatory process, because the regenerative capacity returns before the peak of the inflammatory phase (Bombal *et al.*, 1983). Other aetiological hypothesis are thus proposed:

(1) erythropoietin deficiency similar to that described in burn patients (Robinson *et al.*, 1973);

(2) drug-induced haematological toxicity (Camitta, Storb & Thomas, 1982)

(3) immune mechanisms analogous to those implicated in TEN and comparable to those responsible for the aplastic anaemias seen in GVHR (Camitta *et al.*, 1982).

The neutropenia present in this case upon admission worsened considerably on the fifth day, at which time the neutrophil count continued to decline steadily. The characteristics of this neutropenia are the same as those described in a recent study of 10 cases of TEN (Westly & Wechsler, 1984). In this series, neutropenia appeared between the second and fifth day of hospitalization and was of prognostic value as its persistence or aggravation (as in this case) coincided consistently with fatal outcome in the 10 days following onset, while a restoration to normal was indicative of recovery. Many cases of leukopenia have been noted (Halebian *et al.*, 1983; Westly & Wechsler, 1984) and it is primarily the PMN population that is implicated in the documented cases (Jarkowski & Hartmer, 1962; Mitroshina *et al.*, 1976; Juldashev *et al.*, 1978, quoted by Westly *et al.*, 1984; Haynes, 1978; Finlay, Richard & Holt, 1982; Ortiz, 1982). The aetiology of this TEN-associated neutropenia is as yet undetermined. An increase in the percentage of circulating band cells is seen while sternal puncture, when performed, consistently reveals a hypercellular tissue consisting predominantly of immature elements with a near absence of mature neutrophils. This finding is compatible either with a premature release of the most developed myeloid cells, or with an arrest in the maturational process, or with a recent resumption of granulocytopoiesis.

Low levels of circulating neutrophils cannot be explained by an increased rate of consumption due to infection (Westly & Wechsler, 1984) or gram negative septicemia (Halebian *et al.*, 1983) since there is not always a correlation. Complement dependent leucocytic aggregation seems improbable (Hammerschmidt, Stroncek & Bowers, 1981), as serum complement levels remain normal. A drug-induced toxicity related in our case to the intake of amoxycillin and/or

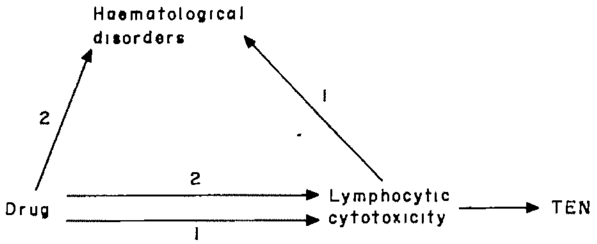

FIGURE 2. Relationship between drug, TEN and haematological disorders. 1, same mechanism. 2, distinct mechanisms.

paracetamol is plausible, since the variability of the marrow cellularity in drug-induced agranulocytosis is well known (Young & Vincent, 1980). The other drugs (chloramphenicol, rifampicin, gentamycin) given during the hospitalization had been started after the neutrophil count fell. It is finally possible that the immune mechanisms that cause the TEN also cause the neutropenia. Lymphopenia is a frequent finding and was present in 24 cases of a series of 25 (Bombal *et al.*, 1983); it appears early, sometimes preceding the advent of the skin symptoms, and is profound (mainly affecting the T-helper cells). It recovers only very gradually. Its precocity and its frequency seem to indicate a relationship with the pathogenic immune mechanisms, comparable to the lymphopenias which are present in the GVHR (Billingham & Streinlein, 1968).

The thrombopenia appears less frequently. In the cases where, as in the one quoted here, it is severe, it could result from the same causes, if DIC has been previously excluded (Kvasnicka *et al.*, 1979).

Two quite different hypotheses may thus explain drug-related haematological disorders (anaemia, neutropenia, lymphopenia, thrombopenia) which are present in TEN (Fig. 2): (1) the drug causes, by a secondary mechanism, the haematological disorders through the same mechanism as the one which leads to the TEN (lymphocytic cytotoxicity); or (2) the drug causes the haematological disorder through a different mechanism (drug-induced haematological toxicity).

The first hypothesis considers a possible role of cellular-mediated mechanisms in the depression of haematopoiesis. The lymphopenia seen in TEN and GVHR shows a severe depletion of T-helper cells (OKT 4) (Roujeau *et al.*, 1983; Atkinson *et al.*, 1982); T suppressor cells (OKT 8) are predominant in the cutaneous lesions of acute GVHR (Saurat & Chavaz, 1983). A disturbance of the OKT 4/OKT 8 ratio with OKT 4 depression and cytotoxic OKT 8 derepression could possibly be part of the pathogenic mechanism implicated not only in TEN and GVHR, but also in their associated haematological disturbances.

TEN-related anaemia and lymphopenia seem to be related to this immune mechanism, in view of their frequency, rapid development, and the existence of similar disturbances in GVHR. The origin of TEN related neutropenia is less clear. Further investigations are necessary to establish, in cases of TEN, the existence of cellular and serum factors capable of inhibiting *in vitro* growth of blood cells precursors, in particular the myeloid (CFU-C) and erythroid (BFU-E and CFU-E) precursors.

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### Lack of effect of topical spironolactone on sebum excretion

MADAM, The anti-androgenic effects of systemically administered spironolactone (SL) have been exploited in treating hirsutism (Boisselle & Tremblay, 1979; Cumming *et al.*, 1982) and, more recently, acne (Goodfellow *et al.*, 1984). In doses of 100–200 mg per day, SL improves acne clinically and lowers sebum excretion rate (SER) by about 50% (Keahey *et al.*, 1983; Burke & Cunliffe, 1985).

Clinical improvement in acne has also been reported with the use of topical formulations of spironolactone (Messina, Manieri & Rizzi, 1983) and its active metabolite, canrenone (Neilsen, 1983) but SER determinations were not done in these studies. If a topical anti-androgen is to be effective, it should produce a fall in SER.

In our study, patients with moderately severe facial acne [Grade 1·0–3·0, assessed by the method of Cunliffe & Burk (1984)] were randomly allocated to three treatment groups. Group 1, eleven patients (four men, seven women), received treatment with 3% spironolactone powder finely ground and evenly dispersed in Unguentum Merck. Group 2, eleven patients (seven men, four women), received treatment with 5% spironolactone cream (SAS 1060, Schiapparelli Farmaceutici S.p.A., Turin, Italy) which had previously been reported to be beneficial in acne by Messina *et al.* (1983), Group 3, nine patients (five men, four women), received treatment with 3% potassium canrenoate solution made up in a cream with 2% lactic acid and Unguentum Merck. Patients in Groups 1 and 3 used a measured amount of the creams, each delivering spironolactone 3 mg or canrenone 0·1 gm, applied to the forehead twice a day. Patients in Group 2 also applied the cream to the forehead twice a day, but used unmeasured amounts from the tube as supplied by Schiapparelli Farmaceutici S.p.A., Turin, Italy. The anti-androgenic effect was tested by measuring SER on the forehead by the method of Strauss and Pochi, as modified by Cunliffe and Shuster (1969), before treatment and after 1 and 2 months of treatment.

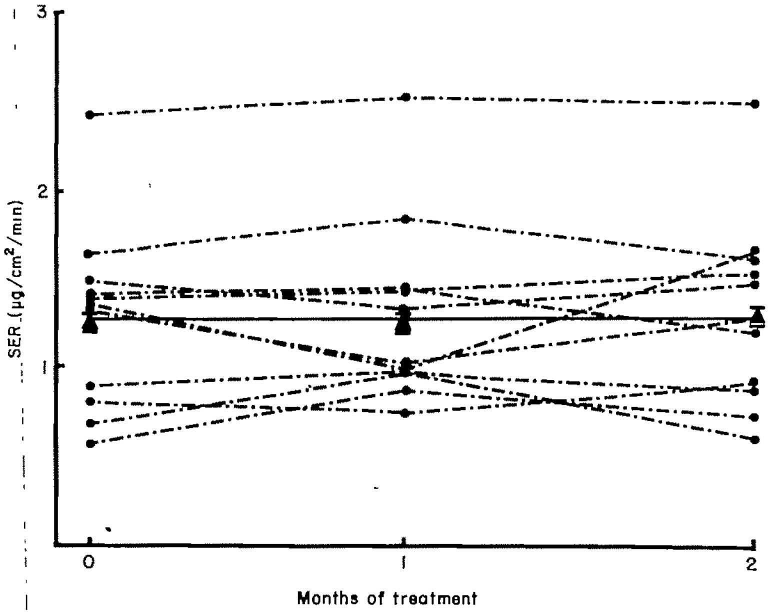

FIGURE 1. SER in 11 patients before, and after one and two months' treatment with topical 3% spironolactone cream —$\bar{\blacktriangle}$— = mean ± SEM.

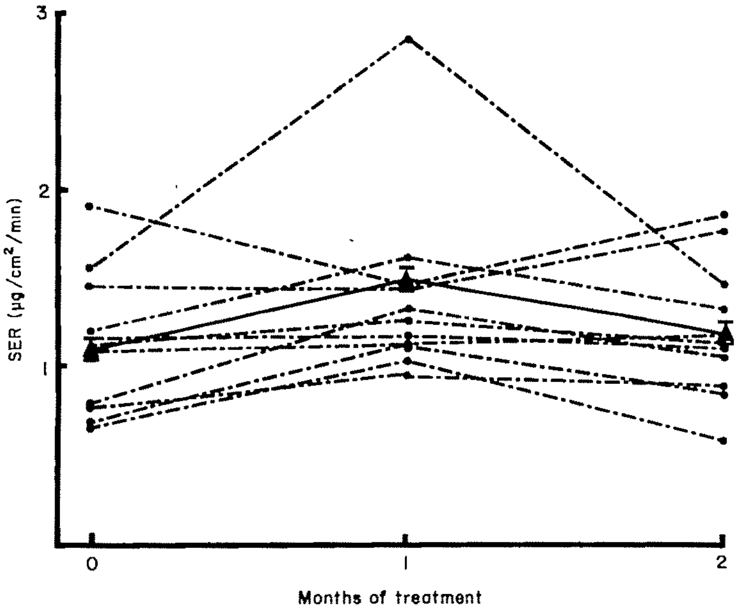

FIGURE 2. SER in 11 patients before, and after one and two months' treatment with topical 5% spironolactone cream. —▲— = mean ± SEM.

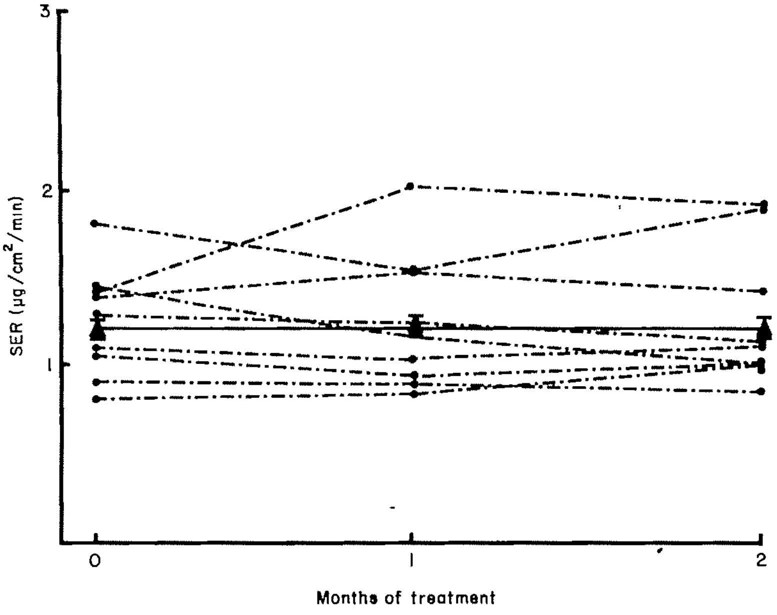

FIGURE 3. SER in nine patients before, and after one and two months' treatment with topical 3% potassium canrenoate cream. —▲— = mean ± SEM.

TABLE Effect of three spironolactone preparations on the sebum excretion rate (SER) in relation to pretreatment SER

| | | Mean SER $\mu$g/cm$^2$/min ± SEM | | |
|---|---|---|---|---|
| SER | No. of patients | Before treatment | After one month of treatment | After two months of treatment |
| High<br>($>1{\cdot}2$ $\mu$g/cm$^2$/min) | 17<br>(11 male, 6 female) | 1·53 ± 0·07 | 1·60 ± 0·12 | 1·57 ± 0·09 |
| Low<br>($<1{\cdot}2$ $\mu$g/cm$^2$/min) | 14<br>(5 male, 9 female) | 0·90 ± 0·04 | 1·05 ± 0·05 | 0·96 ± 0·05 |

The patients treated with 3% spironolactone cream and those treated with 5% spironolactone cream showed no change in their mean SER after two months of treatment ($P>0{\cdot}6$ and $P>0{\cdot}1$, respectively). (Figs. 1 and 2). Nor was there any reduction in SER in the patients using the metabolite preparation, 3% potassium canrenoate cream at the end of the two month period ($P>0{\cdot}8$) (Fig. 3). No significant effect of the drugs on SER was revealed when the results were re-analysed to determine whether patients with a high SER might respond differently to those with a lower SER (Table).

These observations suggest that topical spironolactone is unlikely to be beneficial in treating acne. Possible explanations for the lack of anti-androgenic effect from our topical formulations include:

1. The vehicle used in our study may not have been adequate to deliver the drugs to the target sebaceous glands. This may apply particularly to the spironolactone which we could not dissolve in 5% isopropyl myristate in isopropyl alcohol as used by Weissmann *et al.* (1985). The other SL preparation was the original cream (SAS 1060) as used by Messina *et al.* (1983).

2. The anti-androgenic effect of SL may be mostly due to its effects on production and plasma protein binding of sex hormones, rather than its effect at the target site.

3. *Propionibacterium acnes* which is almost unique to man may metabolize the topically applied anti-androgen.

To date, topical formulations of systemically effective anti-androgens (i.e. cyproterone acetate) have been generally disappointing in their effects in man (Cunliffe & Simpson, 1980).

The success of topical spironolactone in reducing sebaceous gland size in hamsters offers hope for successful topical therapy (Weissmann *et al.*, 1985). Search for a more effective vehicle is one avenue that merits further exploration.

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## Bullous amyloidosis

Madam, We have read the report by Ruzicka *et al.* (*British Journal of Dermatology*, 1985, **113**, 85) of their patient with a relapsing bullous dermatosis with amyloid deposits in the upper dermis.

Their table of previously reported cases does not include ours (Walton *et al.*, 1983) and we mention it for completeness. In our patient the blister was at the level of the lamina lucida, although the amyloid deposits that were demonstrated were considerably deeper than this in the blood vessels of the dermis. No immunoreactants were demonstrated in involved or clinically uninvolved skin. The blisters were mainly trauma induced and we suggested that, as they did not appear to be directly related to the amyloid, they involved a change, as yet unidentified, in the skin collagen.

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## Dermatitis herpetiformis

MADAM, We read with interest the paper by Griffiths, Leonard and Fry (*British Journal of Dermatology*, 1985, **112**, 443) describing the exacerbation of dermatitis herpetiformis (DH) by indomethacin. It has also been our experience that non-steroidal anti-inflammatory drugs cause a worsening of DH in patients treated only with dapsone. However, one female patient controlled by a gluten free diet alone, experienced no exacerbation of her DH when given indomethacin for menstrual problems.

Of particular interest to us has been the possible role of non-steroidal anti-inflammatory drugs (NSAIDs) in precipitating or worsening other bullous dermatoses in the elderly. We report two cases of benign mucous membrane pemphigoid (BMMP) which appear to have been precipitated by indomethacin. The first, a 78-year-old man who had suffered from rheumatoid arthritis for 40 years, developed blisters in the mouth and on the trunk and limbs two months after commencing indomethacin therapy. The clinical diagnosis of BMMP was confirmed by histology and direct and indirect immunofluorescence studies. Within a week of stopping the indomethacin therapy the oral lesions had completely cleared. Oral steroid therapy was then commenced to accelerate resolution of the lesions on his legs, as he was awaiting hip replacement. Control was achieved within a week, on prednisolone 12·5 mg daily.

The second patient, a man aged 72 years, had suffered from rheumatoid arthritis for four years. He complained of intermittent blistering on the legs for four years, sore eyes for eleven months, and blisters in the mouth for five months. His blisters had started six weeks after indomethacin therapy was instituted, and he had classical ocular, oral and cutaneous lesions of BMMP. Within 10 days of stopping indomethacin his vision had improved from counting fingers to reading large print. His other lesions were controlled within two weeks, during which time the Rheumatologist added prednisolone 5 mg daily for his arthritis.

Both patients attributed their skin disease to their indomethacin therapy, and certainly they both improved considerably when the drug was stopped, and later stayed clear on a relatively low dose of oral prednisolone. As patients who develop BMMP and bullous pemphigoid are in an age group who suffer from arthritis, the general practitioners of five BMMP patients and 25 bullous pemphigoid patients were contacted to ascertain the drug histories of the patients in the six months prior to the appearance of the bullous dermatoses. All five BMMP patients had taken a NSAID in the six months prior to the onset of their eruption. Twenty-two replies were received regarding the bullous pemphigoid group and, of these, 11 patients had received NSAIDs in the six months prior to development of their eruption

Bullous eruptions are reported to occur more commonly in patients already suffering from an auto-immune disorder (Chlorzelski, Jablonska & Blaszczyk, 1968; Jordan *et al.*, 1969; Mackie, Saikia & Morley, 1973; Obasi & Savin, 1977) and pemphigoid is said to be associated with rheumatoid arthritis (Cullen *et al*, 1978). However, it is our impression that drugs may play an important role in the precipitation of bullous dermatoses rather than there being a true disease association. This theory is supported by a recent report of a case of anogenital cicatricial pemphigoid induced by clonidene (Van Joost, Faber & Manuel 1980).

A possible mechanism for such an event might be that drug induced metabolic disturbance at a local level at the site of the basal cells leads to the formation of immunogens and is one of the initiating factors in sub-epidermal bullous dermatoses. There is indeed evidence of the presence of basal cell cytoplasmic auto antibodies in the sera of patients with drug-induced eruptions (Van Joost, 1974, 1979). A second possible explanation comes from the finding that E series prostaglandins inhibit the expression of Ia antigens by murine macrophages and human Langerhans cells (Snyder, Beller & Unanne, 1982; Berman *et al*, 1985). Drugs such as indomethacin, which inhibit prostaglandin synthesis have been shown to increase Ia expression by these cells, mimicking the action of Ia-inducing lymphokines such as gamma interferon. The quantitative level of Ia expression may be important in regulating immune responses; it has been suggested that increased Ia expression enhances immunoglobulin production (Haneway *et al.*, 1984), and may lead to the generation of auto-immune reactions (Bottazzo *et al.*, 1983). Thus, indomethacin and other cyclo-oxygenase inhibitors might exacerbate autoimmune disease through this mechanism. In this context, it is interesting that indomethacin has been reported to stimulate auto-antibody synthesis in mice (Haneway *et al.*, 1984).

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## Reply

MADAM, We too have noticed that dermatitis herpetiformis (DH) patients controlled entirely on a gluten free diet (GFD) do not seem to suffer an exacerbation of their disease when given non-steroidal anti-inflammatory drugs. This may be analogous to the findings of Haffenden *et al.* (1980) who reported that potassium iodide patch testing will induce DH lesions in patients on sulphone treatment alone but not in those patients controlled on a GFD. Maybe chemo-attractants in the skin of these patients decline below a certain threshold as a result of gluten no longer acting as an antigenic stimulus.

There is a strong link between benign mucous membrane pemphigoid (BMMP) and rheumatoid arthritis, this could be as a result of NSAID administration. Shuttleworth and Graham-Brown (1985) have described BMMP occuring with D-penicillamine treatment. Autoimmune disorders do seem to occur together and it seems more likely that the drugs implicated act as a co-factor with rheumatoid arthritis rather than the drug alone being responsible.

It would of course be simplistic to assume that any pathogenic mechanism for the appearance of skin lesions in DH and their exacerbation by NSAIDs is anything but multifactorial, involving an interaction between immunological and chemical processes. The increase of Ia expression by NSAIDs would be in accord with this concept in relation to both DH and psoriasis.

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## Erythromelanosis follicularis faciei in women

MADAM, Kitamura *et al.* (1960) described a new dermatosis characterized by a sharply demarcated reddish brown discolouration of the face and neck together with tiny follicular papules. They described the syndrome in six male Japanese subjects and named it 'Erythromelanosis follicularis faciei' (EEF). Mishima and Rudner (1966) reported four male caucasian subjects with the syndrome. The first and only woman with EFF was described by Andersen (1980). Since then, two additional males with the syndrome have been described (Watt & Kaiser, 1981; Borkovic, Schwartz & McNutt, 1984). From these reports one would conclude that EFF is mainly a disease of men (12 out of 13 patients). We have recently seen five patients, all females, with typical EFF. Their age range was 15–22 years. Four patients had had the eruption since infancy and one since adolescence. All the patients had pronounced keratosis pilaris on the upper extremities. Biopsy was performed in one patient and the histological examination was not diagnostic. However, direct immunofluorescence examination revealed granular deposition of IgM in the dermo-epidermal junction.

Our observation indicates that EFF is also a dermatosis of women. Since it is mainly a cosmetic problem it needs no treatment. Most of the patients previously reported, as well a those described here, had suffered from the eruption since infancy or adolescence. In our opinion EFF is overlooked by many physicians and is much more common in the population than is realized. The more the syndrome becomes familiar to the dermatologist the fewer patients will be misdiagnosed.

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## Megaloblastic anaemia and methotrexate treatment

MADAM, I read with great interest the important observations of Dodd, Kirby and Munro (1985) concerning the occurrence of folate deficient megaloblastic anaemia during methotrexate therapy for psoriasis. I have recently had two similar cases who developed an acute megaloblastic anaemia during methotrexate therapy, and the history of one of these suggested a possible explanation for the sudden development of the anaemia which has important implications for dermatologists using this drug.

A 64-year-old, obese woman with severe psoriasis had been well controlled on 15 mg methotrexate once weekly for six years with no hepatotoxicity or marrow depression. It was then found that her mean corpuscular volume had risen to 112 fl from normal levels although her haemoglobin and serum folate were normal. Eight weeks later her haemoglobin had fallen to 6·0 g/dl, white cell count $2{\cdot}6 \times 10^9$/l, platelets $75 \times 10^8$/l, serum folate 1·2 pg/ml (normal 2·2–12 pg/ml), serum B12 normal. A bone marrow aspiration confirmed a severe megaloblastic anaemia. Careful questioning revealed that she had, unknown to her doctors, gone on a severe weight-reducing diet consisting mainly of apples (a very poor source of folate) over the preceeding three months. She was treated with folinic acid and blood transfusion but she developed a severe bronchopneumonia, probably resulting from the leucopenia, and she died.

The body reserves of folic acid are very limited and dietary depriviation may deplete these after only a few months. Methotrexate therapy has been shown to lower the erythrocyte folate concentration during long term therapy (Hendel & Nyfors, 1985). If a patient is receiving a folic acid antagonist such as methotrexate and goes on a very restricted diet the risk of folate deficiency and megaloblastic anaemia is greatly enhanced. This risk is probably increased in the elderly since many already exist on a relatively poor diet.

I suggest that all patients, especially the elderly, who are receiving methotrexate should be encouraged to maintain a proper diet during treatment. In particular, they should be warned not to embark on any special diets without prior discussion with their doctor or dietitian. Failure to do so could have potentially fatal consequences as this case illustrates.

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MADAM, Dodd, Kirby and Monroe (1985) recently reported four cases of megaloblastic anaemia in psoriatic patients receiving long-term weekly methotrexate (MTX) therapy. The authors suggested that MTX treatment should be withdrawn if the MCV exceed 106 fl, in order to reduce the incidence of severe anaemia.

We would like to report a case of severe megaloblastic anaemia in a patient treated with MTX. The patient was a 47-year-old woman with a twenty year history of psoriasis. For 13 years she had been treated with weekly oral doses of MTX without any complications. During the last 6 months the MTX dose given was 15 mg weekly. The patient had undergone repeated liver biopsies but no sign of cirrhosis had been demonstrated. During the last 3 months a severe anaemia developed rapidly with the level of haemoglobin falling from 9·1 mmol/l to 4·2 mmol/l. Bone marrow examination revealed megaloblastic anaemia, and erythrocyte folate was decreased to <2·3 nmol/l (normal 9–41 nmol/l). The megaloblastic anaemia was complicated by a moderate haemolysis. As any other cause of the macrocytic anaemia could be excluded, MTX was considered to be the causative agent.

The patient recovered after the withdrawal of MTX and treatment with blood transfusion and oral folate. However, the severe psoriasis relapsed, and we therefore resumed treatment with a dosage of MTX of 15 mg weekly supplemented by 15 mg oral folate given daily except on the days of MTX treatment. The patient has since been followed up monthly and over the subsequent 5 months the levels of MCV, erythrocyte folate and haemoglobin have all been normal.

Hendel and Nyfors (1985) provide evidence that periodical control of erythrocyte folate concentration, using the pre-therapeutic value as a reference point, is a suitable method of revealing even slowly proceeding folate depletion under MTX treatment. The haemoglobin and MCV do not change before a pronounced state of folate deficiency has developed. As MTX is of great importance in cases of severe psoriasis we do not advise withdrawal of the drug on finding falling levels of erythrocyte folate or macrocytosis, but instead, when MTX is given weekly, we recommend supplementation with orally administered folate on the days when the patient does not receive MTX. In addition, erythrocyte folate estimation should be included in the monitoring of patients on long-term MTX therapy.

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*British Journal of Dermatology* (1986) **114**, 270.

# *Book Reviews*

**Chemical Safety Regulation and Compliance.** Edited by F.Homburger and J.K.Marquis (1985) Basel: Karger. Pp. 129. price SFr 115.

The work provides simple clear explanations of the complexities of several aspects of American laws and policies to regulate exposure to harmful chemicals in water, food, air and in factories. Although the book mainly adheres to regulatory requirements, there are some passages of honest self-doubt. One such example is the intense pre-occupation with a three-step regulatory process in assessing food safety, while at the same time the requirements may fail to protect the public from other food hazards because they are not defined (p. 39). The frustrations of the Delaney Clause in relation to any food additive and carcinogenic risk are recognized. The mathematical approach to risk assessment of chemical mixtures reads as though biological activities were to be regulated; they have not found favour in Europe.

W.E.Parish

**1985 Yearbook of Dermatology**. Edited by Arthur J.Sober and Thomas B.Fitzpatrick. Chicago: Yearbook Medical Publishers Inc. Price £43.00.

The 85th edition of the yearbook begins with a number of statistics and I will therefore do the same. We have 22 chapters covering a large number of articles from 102 Journals, all but four in English. In addition, 76 review articles are mentioned at the beginning of the book. There are two editors and eleven contributing editors. The chapters, as expected, cover a wide range of dermatological topics; the largest two being devoted to malignant melanoma (30 papers discussed) and non-melanoma skin cancer (43 papers discussed).

Those dermatologists who have a regular subscription to the yearbook will be pleased to see it in its usual recognizable and easily readable form. Those who do not subscribe, but who might be persuaded to do so, should consider the fact that in the Journals perused, the gastroenterological, ophthalmological, obstetric and other literature is covered. I am sure that this is one of the great strengths of the Yearbook and would personally like to see greater emphasis on abstracts from the non-dermatological literature, as I am sure that the bulk of the purchasers of the Yearbook will be regular readers of the conventional dermatological literature.

At £43.00 for 462 pages, excluding the index, the book is good value for money.

Rona M.Mackie

**Control of Sexually Transmitted Diseases.** World Health Organisation 1985 Geneva: H.M.S.O. Pp. 110. Price £5.75. (paperback)

More people are at risk of contracting sexually transmitted diseases than ever before, and the social and economic consequences of these diseases are an increasing problem world wide. Control efforts are often unsatisfactory because of bad organization rather than shortage of resources. This book contains an outline of national control strategies and was produced following a WHO Scientific Working Group in 1982. It covers planning, screening, treatments, training and evaluation. The approach is very abstract, intended to provide a theoretical framework, but making it difficult to relate to practical action, and sometimes lapsing into bland platitudes. The lists of references are good and to some exent overcome this, but demonstrate the lack of data from the countries for which the book is intended. It will not tell anyone how to start a disease control programme, but it will give a general guide as to whether they are proceeding in the right direction.

M.R.Fitzgerald

*British Journal of Dermatology* (1986) **114**, 271.

# *News and Notices*

### First European Congress of Photobiology—Preliminary Announcement

The first European Congress of Photobiology will take place from 7 to 12 September 1986 in Grenoble, France. Further details may be obtained from: Dr J.Cadet, (local organiser), Centre d'Etudes Nucleaires, DRF- Documentation Chimie 85X, F. 38041 Grenoble, France.

### Skin Pharmacology Society Third Annual Symposium

The Skin Pharmacology Society will hold its third Annual Symposium on Friday and Saturday, 1 & 2 August 1986, at the Karolinska Institute in Stockholm, Sweden. A major topic will be: 'Pharmacology of the Cells of the Dermo-Epidermal Junction'. On 31 July 1986, the Society will also co-sponsor a special mini-symposium in association with the III World Conference on Clinical Pharmacology and Therapeutics entitled: 'Skin as a Response Organ for Drugs'. Invited speakers and round-table discussions will highlight the programme. Free communications are strongly encouraged. For further information and abstract forms, please write to: Osten Hagermark, M.D., Ph.D., Department of Dermatology, Karolinksa sjukhuset, S-104 01, Stockholm, Sweden.

### VIIIth International Congress of Dermatologic Surgery

The British Dermatological Surgery Group is hosting this annual event on behalf of the International Society for Dermatologic Surgery and its various affiliated societies throughout the world. The conference will be held from 21 to 24 September 1986 at the City University, London. Further information regarding the scientific programme can be obtained from Dr R.P.R.Dawber, Department of Dermatology, The Slade Hospital, Headington, Oxford, U.K. Travel and accommodation details are available from Mrs I.Green, Congress Secretariat, VIP International Ltd., 42 North Audley St, Grosvenor Square, London W1A 4PY, U.K.

### Annual Meeting of the British Society for Investigative Dermatology, 1986

The next Annual Meeting of the B.S.I.D. will be held at York University, on the 23 and 24 September 1986. The scientific progamme will include free communications (oral and poster), guest lectures and workshops. The Meeting is open to non-members of the Society. Abstracts must be received by Dr R.D.R.Camp by 13 June 1986 and should be submitted on the official forms. For further information and abstract forms, please write to: Dr R.D.R.Camp, Chairman, B.S.I.D., Institute of Dermatology, Homerton Grove, London E9 6BX, U.K.

*British Journal of Dermatology* (1986) **114**, 272.

# *Erratum*

White, M.I. & Noble, W.C. (1985) The cutaneous reaction to staphylococcal protein A in normal subjects and patients with atopic dermatitis or psoriasis. *British Journal of Dermatology*, **113**, 179.

Would readers please note that Table 1 on page 180 of the above issue was published incorrectly. The correct table appears below.

TABLE 1. Response to intradermal injection of 5 ng protein A

| 15 min | Weal (mm ± SD) | | Erythema (mm ± SD) | |
|---|---|---|---|---|
| Normal volunteers (12F, 13M) | 9·0 ± 4·3 | ** | 28·5 ± 10 | *** |
| Atopic dermatitis (8F, 10M) | 7·5 ± 1·8 | | 9·9 ± 4·5 | |
| Psoriasis (9F, 10M) | 7·6 ± 2·5 | | 23·3 ± 7·5 | |
| **6 h** | **Erythematous papules (mm ± SD)** | | | |
| Normal volunteers (8F, 8M) | 8·75 ± 9·8 | NS | | |
| Atopic dermatitis (8F, 9M) | 9·2 ± 9·2 | | | |
| Psoriasis (6F, 8M) | 13·7 ± 8·2 | | | |
| **24 h** | **Indurated erythema (mm ± SD)** | | | |
| Normal volunteers (9F, 8M) | 33·5 ± 22·2 | *** | | |
| Atopic dermatitis (8F, 9M) | 17·0 ± 17·9 | | | |
| Psoriasis (9F, 9M) | 37·6 ± 24·0 | | | |
| **48 h** | **Erythema (mm ± SD)** | | | |
| Normal volunteers (8F, 8M) | 2·5 ± 2·6 | *** | | |
| Atopic determatitis (7F, 7M) | 18·4 ± 23·6 | | | |
| Psoriasis (8F, 7M) | 45·7 ± 42·3 | | | |

Compared by analysis of variance.
*** $P < 0{\cdot}001$.
** $P < 0{\cdot}01$.
NS $P > 0{\cdot}05$.

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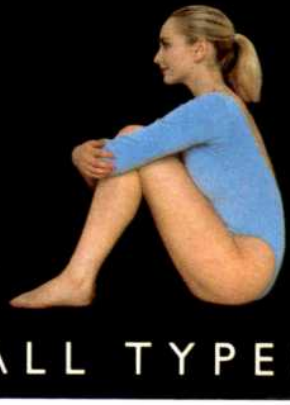

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## Departments of Dermatology and Radiation Oncology

11–12 April 1986

SYMPOSIUM ON MALIGNANT DISEASE AND THE SKIN AT THE UNIVERSITY OF LIVERPOOL

Subjects include epidemiology, pathogenesis and therapy.

Speakers include Prof. J A A Hunter, Edinburgh, Dr John White, Southampton, Dr Neil Smith, London, Prof. Wilson-Jones, London, Dr A Chu, London, Dr S S Bleehen, Sheffield, Dr Margaret Spittle, London.

Accommodation: University Hall of Residence

Registration Fee: £25.00

Details from Prof. C F H Vickers, Dept. of Dermatology, Royal Liverpool Hospital—or Dr M J Garrett, Dept. of Radiation Oncology, Clatterbridge Hospital, Bebington, Wirral

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The *British Journal of Dermatology* publishes original articles on all aspects of the normal biology, and of the pathology of the skin. Originally the Journal, founded in 1888, was devoted almost exclusively to the interests of the dermatologist in clinical practice. However, the rapid development, during the past twenty years, of research on the physiology and experimental pathology of the skin has been reflected in the contents of the Journal, which now provides a vehicle for the publication of both experimental and clinical research and serves equally the laboratory worker and the clinician. To bridge the gap between laboratory and clinic the Journal publishes signed editorials reviewing, primarily for the benefit of the clinician, advances in laboratory research, and also reviews of recent advances in such aspects of dermatology as contact dermatitis, therapeutics and drug reactions. Other regular features include book reviews, correspondence and society proceedings. The Journal is the official organ of the British Association of Dermatologists and also of the Netherlands Association for Dermatology and Venereology, but it attracts contributions from all countries in which sound research is carried out, and its circulation is equally international.

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| | | | | | |
|---|---|---|---|---|---|
| gram(s) | g | minute(s) | min | molar | mol l |
| kilogram(s) | kg | centimetre(s) | cm | millilitre(s) | ml |
| milligram(s) ($10^{-3}$g) | mg | second(s) | s | gravitational acceleration | *g* |
| microgram(s) ($10^{-6}$g) | μg | cubic millimetre(s) | $mm^3$ | micrometre(s) | μm |
| nanogram(s) ($10^{-9}$g) | ng | millimetre(s) | mm | per cent | |
| picogram(s) ($10^{-12}$g) | pg | millicurie(s) | mCi | isotopic mass number placed as | $^{131}$I |
| hours(s) | h | milliequivalent | mmol | | |

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The British Journal of Dermatology is owned by and is the official organ of the British Association of Dermatologists. It is also the official organ of the Nederlandse Vereniging voor Dermatologie en Venereologie (Netherlands Association for Dermatology and Venereology).

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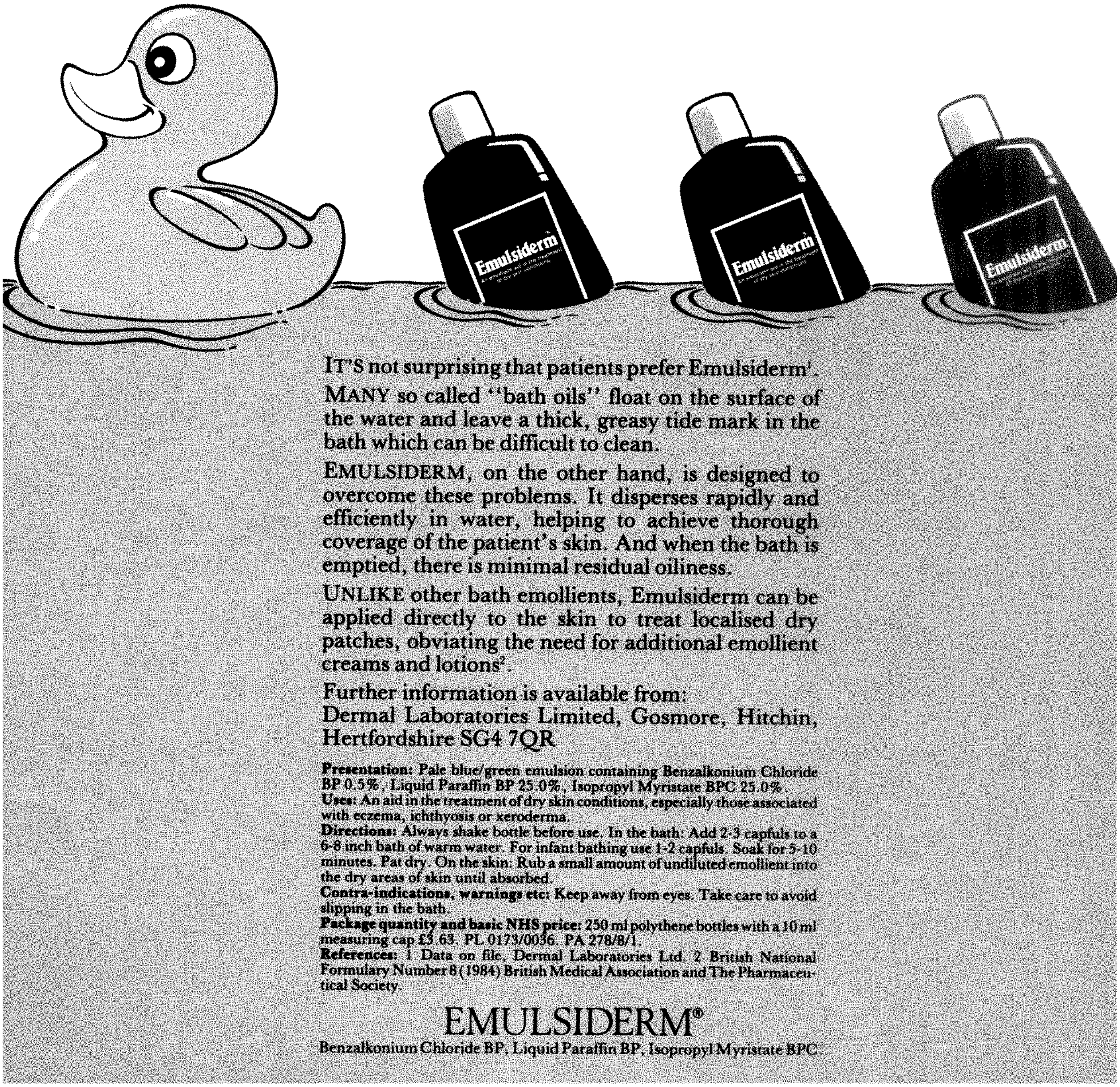

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**Presentation:** Pale blue/green emulsion containing Benzalkonium Chloride BP 0.5%, Liquid Paraffin BP 25.0%, Isopropyl Myristate BPC 25.0%.
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**Contra-indications, warnings etc:** Keep away from eyes. Take care to avoid slipping in the bath.
**Package quantity and basic NHS price:** 250 ml polythene bottles with a 10 ml measuring cap £3.63. PL 0173/0036. PA 278/8/1.
**References:** 1 Data on file, Dermal Laboratories Ltd. 2 British National Formulary Number 8 (1984) British Medical Association and The Pharmaceutical Society.

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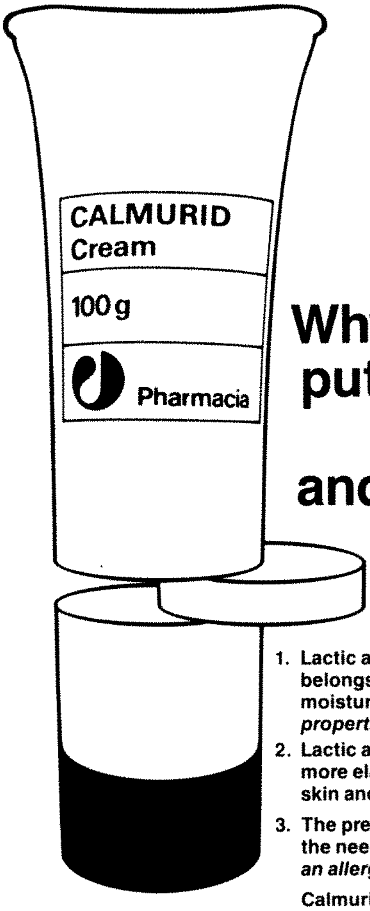

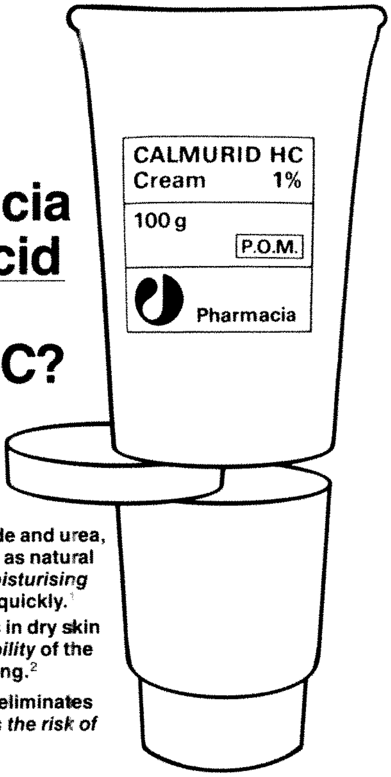

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**CALMURID HC** ***Presentation*** Cream containing 10% urea, 1% hydrocortisone. 5% lactic acid in an emulsifying base. ***Uses*** Atopic eczema, allergic eczema and other inflammatory skin conditions accompanied by hyperkeratosis. ***Dosage and Administration*** Apply twice daily after washing and drying. ***Contra-indications, warnings etc. Adverse Effects*** Calmurid HC can cause smarting temporarily when applied to raw or fissured areas. Topical steroids should not be used extensively in pregnancy, i.e.: in large amounts or for long periods. In infants: long term continuous topical therapy should be avoided. Adrenal suppression can occur even without occlusion. ***Contra-indications*** Skin tuberculosis, viral infections accompanied by dermal manifestations, e.g.: herpes simplex, vaccinia, chicken pox, measles. Syphilitic skin lesions. ***Pharmaceutical Precautions*** Store in a cool place but do not freeze. Do not put in alloy containers. ***Legal Category*** P.O.M. ***Package Quantities and Prices (May 1985)*** Tubes of 30g (£2.33). Tubes of 100g (£6.25). ***Further Information*** Calmurid HC is a pleasant cream which does not contain lanolin or preservatives. This results in a negligible risk of allergic reactions and thus good patient compliance. ***Product Licence Number*** 0009/5003R.

***References*** 1 Middleton J.D., Brit. J. Derm. **80**, 437 (1968). 2 Middleton J.D., J. Soc. Cosmet Chem. **25**, 519-534 (1974). Van Scott, E.J. and Ruey J., J. Am Acad. Dermatol. 11: 867-879 (1984).

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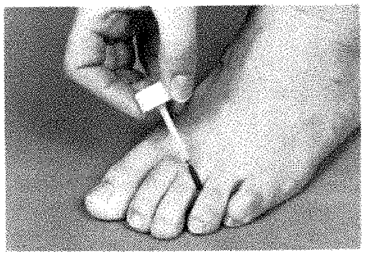

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**Presentation:** A colourless, rapidly drying, non-greasy paint containing boric acid 2%, chlorbutol 3%, methyl undecylenate 5%, propyl undecylenate 1%, salicylic acid (free and methyl ester) 31%

**Uses:** Monphytol is indicated for chronic paronychia, intertrigo, erosio interdigitalis, tinea pedis, tinea circinata, tinea unguium.

**Dosage and administration:** Four times a day, moisten brush with Monphytol and apply to the affected parts, reaching gently into the folds of the skin. Treatment should be repeated from time to time after the condition has subsided to prevent reinfection

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**Pharmaceutical precautions:** Store away from heat

**Legal category:** P

**Package quantities:** Bottles containing 18 ml with brush fitted in cap

**Further information:** Nil

**Product licence number:** 0118/5010

**Basic NHS price:** £0 57 per 18 ml bottle.

*Reference:*

1 Krebs HA, Wiggins D, Stubbs M, Sols A, Bedoya F. Studies on the mechanism of the anti-fungal action of benzoate. Biochem J 1983; **214**: 657-63

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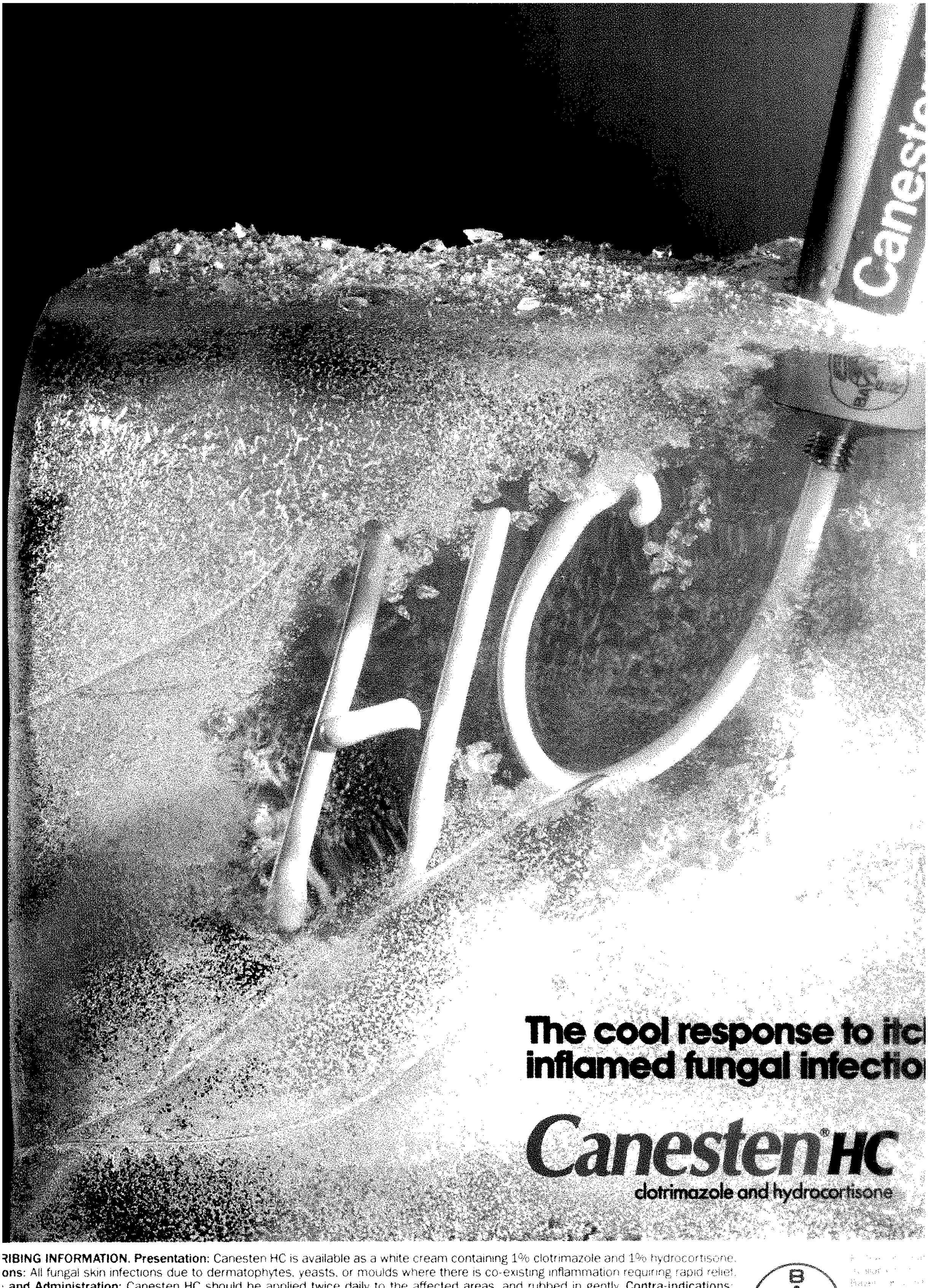

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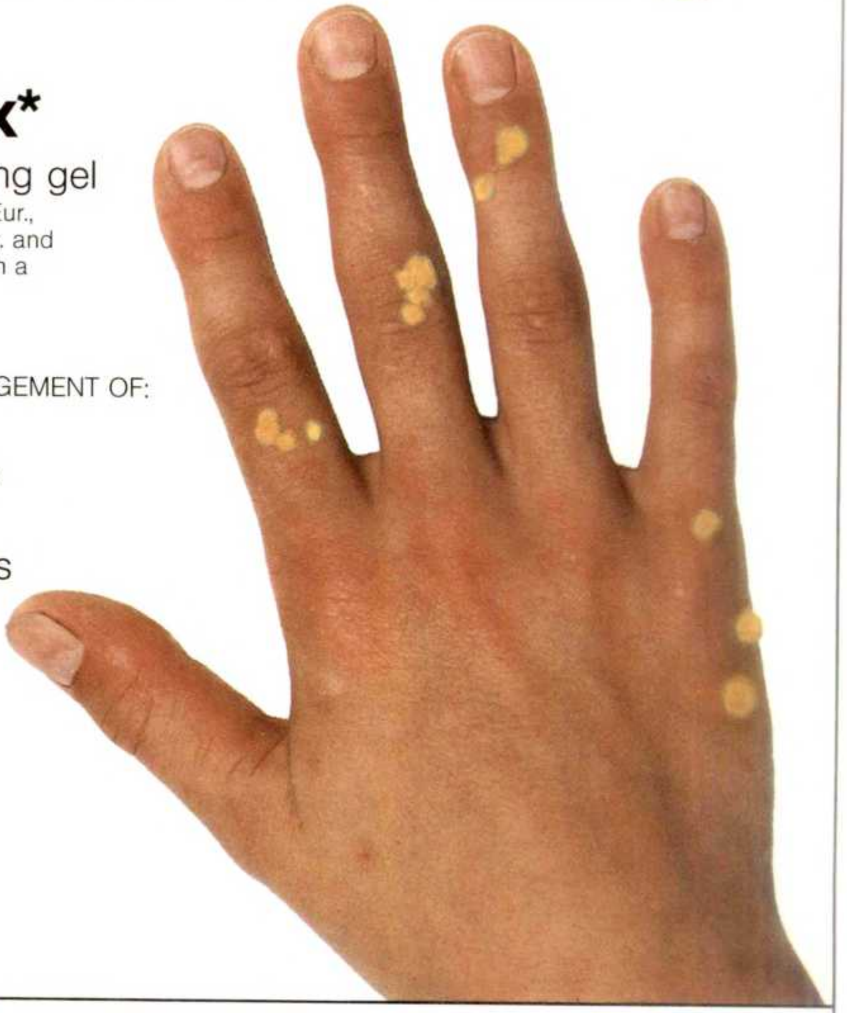

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**Uses** Topical treatment of common, juvenile, plantar and mosaic warts; corns and callouses.
**Contra-indications, warnings etc** Do not apply to facial or anogenital warts. Avoid contact with the eyes. Only apply to the affected area. Keep away from naked flames.
**Pharmaceutical Precautions** Store in cool place. Highly inflammable.
**Legal Category** P
**Package Quantities** 5g Tubes
**Further Information** Warts are contagious and any person suffering from warts should always use their own towel. Most warts will disappear after 6 to 12 weeks of treatment with Cuplex gel, providing instructions are carefully and consistently followed. Where, however, the wart continues to increase in size after 6 weeks treatment and the patient has not consulted a doctor, the patient should be advised so to do.
**Product Licence Number** 0033/0100
**Basic NHS price** 5g gel £1.83

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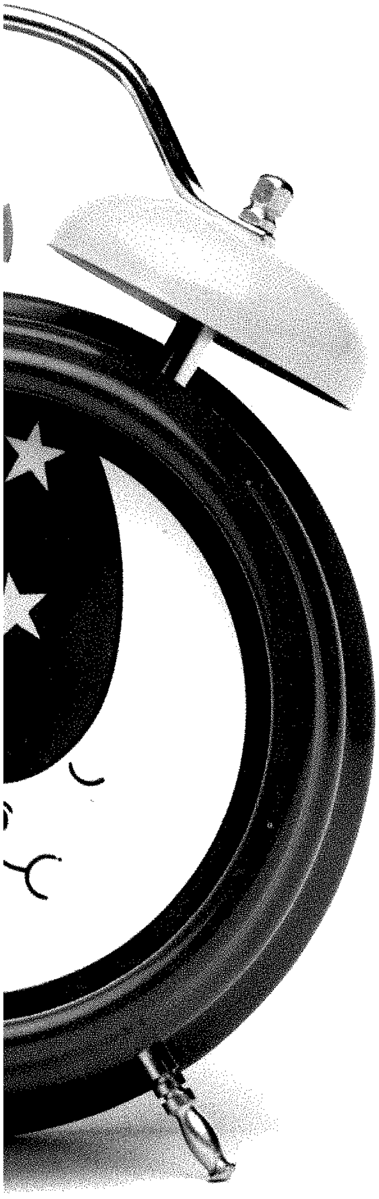

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# Effect of recombinant alpha A-interferon on DNA synthesis and differentiation of human keratinocytes *in vitro*

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## SUMMARY

Interferons are known to protect cells against virus infection, to inhibit cell growth in a number of cell lines and also to modulate cell differentiation. Their possible clinical application has now entered its final phase. The aim of this investigation was to study the effect of recombinant $\alpha$ A-interferon ($r$ IFN-$\alpha$ A) on human keratinocyte DNA synthesis and differentiation *in vitro*. [$^3$H]-TdR incorporation into DNA was used to quantify DNA synthesis in keratinocyte cultures at varying concentrations of $r$IFN-$\alpha$ A. Cell differentiation was determined using polyacrylamide gel electrophoresis of six specific protein fractions extracted from the keratinocytes. Our results indicate that $r$IFN-$\alpha$A is a potent inhibitor of keratinocyte DNA synthesis *in vitro*; it also decreases significantly the amount and synthesis of non-covalently bound keratins while increasing the disulphide cross-linked keratins. No qualitative changes in keratinocyte proteins were observed.

Over the last decade it has become clear that interferons may alter several cellular mechanisms (Stiehm, 1982). They are known to inhibit the growth of a number of normal and transformed cell lines *in vitro* and *in vivo* (Borden, Hogan & Voelkel, 1982; Fleischmann, 1982; Harada *et al.*, 1983; Hokland *et al.*, 1983; Quesada *et al.*, 1984). Their effects also include regulation of the expression of specific genes (Clemens & McNurlan, 1985), modulation of cell differentiation (Dolei *et al.*, 1982) and activation of certain cell types in the immune system (Nakamura *et al.*, 1984). Little is known regarding the interaction of recombinant $\alpha$ A-interferon ($r$ IFN-$\alpha$ A) with normal human keratinocytes. In view of its possible clinical application, for example, in treating human papillomas, the present study was undertaken to elucidate the action of $r$ IFN-$\alpha$ A on growth and differentiation of epithelial tissue *in vitro*.

## METHODS

### *Skin specimens*

Keratome biopsies were taken from the buttock or upper thigh of healthy volunteers.

### *Human keratinocyte cultures*

Normal human keratinocyte cultures were established using the procedure reported by Liu and Karasek (1978). A keratome biopsy was incubated with 0·25% trypsin in PBS (pH 7·2) for 60 min at 37°C. After aspiration of trypsin, minimal essential medium (MEM) with 10% foetal bovine serum (FBS), 50 IU/ml penicillin, 50 $\mu$g/ml streptomycin and 50 $\mu$g/ml fungizone was added. Then the epidermis was separated from the dermis, and the epidermal cells released into the medium by gently scraping and agitating both the epidermal and the dermal component of the biopsy. Epidermal cell suspensions ($1{\cdot}5 \times 10^6$ typan blue-excluding cells per ml) were plated on 16 mm culture dishes pre-coated with a collagen type 1 gel (Vitrogen, Flow Laboratories). Cells were incubated at 37°C in 100% humidity in a 95% air/5% $CO_2$ enivronment. After 24 h, the plating medium was replaced with McCoy's 5A medium supplemented with 50 IU/ml penicillin, 50 $\mu$g/ml streptomycin, 50 $\mu$/ml fungizone, $4 \times 10^{-4}$ M L-serine and 10% FBS.

### *Addition of recombinant α A-interferon*

*r* IFN-α A (Ro 22-8181, Roferon-A), kindly provided by the Hoffmann La Roche Company (Basel), was dissolved in distilled water and small volumes kept, with minimal or no airspace, at −30° or −70°C until used. These were added to the basal cell monolayers one week after plating and with each subsequent medium change. Distilled water in growth medium was used as a vehicle control. Doses of *r* IFN-α A between 50 and 800 IU/ml were used, which corresponded to levels of natural interferon found *in vivo*.

### *[$^3$H]-Thymidine labelling of DNA*

Keratinocyte cultures are terminally pulse-labelled for 6 h with 1 $\mu$Ci/ml of [$^3$H]-TdR and the DNA extracted and counted as previously described by Marcelo *et al.* (1978).

### *Differentiation of keratinocyte cultures*

The cultures were serially extracted using four buffers to yield six fractions as described by Marcelo and Tong (1983). The fractions were: (a) keratohyalin granule (KG) fractions (macro-aggregates and high-salt soluble proteins) ($R_2$, $S_2$); (b) SDS soluble proteins ($S_3$); (c) non-covalently cross-linked fibrous proteins ($S_4$); (d) disulphide cross-linked fibrous proteins ($S_5$) and (e) cell envelopes ($R_5$). All the supernates were dialysed at 4°C against distilled water containing PMSF and then lyophilized. Triplicate 10 $\mu$l aliquots of each fraction were assayed by a modified Lowry technique for protein concentration using serum albumin as a standard. Aliquots containing 10–100 $\mu$g of protein were analysed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Separating gels of 12·5% polyacrylamide were used routinely.

## RESULTS

To determine the effect of *r* IFN-α A on DNA-synthesis, interferon was added to one-week-old cultures of normal keratinocytes for 10 days. *r* IFN-α A was added with fresh medium every second day, at doses between 50 and 800 IU. Keratinocytes grown in the presence of *r* IFN-α A for 10 days showed a statistically significant decrease in DNA-synthesis as determined by the incorporation of [$^3$H]-TdR into DNA. With 50 IU/ml there was a 50% decrease and with 800 IU/ml a 90% decrease ($P < 0{\cdot}01$ Mann-Whitney test) (Fig. 1).

In separate experiments, human epidermal keratinocyte cultures were grown in McCoy's 5A medium supplemented with 400 IU/ml *r* IFN-α A for 14 days, and the cultures were then

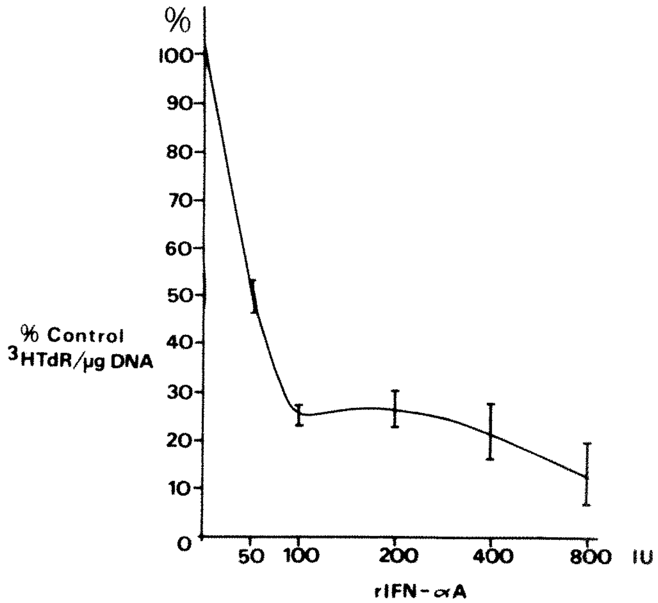

FIGURE 1. Effect of *r* IFN-αA on human epidermal keratinocyte DNA-synthesis. The results are measured as cpm of [$^3$H]-TdR label per µg DNA and are presented as a percentage of the vehicle control. Points are means (± SD) of 6 determinations.

serially extracted in buffers. Table 1 shows that KG-macro-aggregates, KG-supernates and sodium dodecyl sulphate soluble proteins were slightly inhibited by *r* IFN-α A. Non-disulphide cross-linked keratins were markedly inhibited by *r* IFN-α A whereas the disulphide cross-linked keratins were markedly stimulated. Figure 2 shows an SDS-PAGE gel of keratohyalin-granule associated proteins and fibrous proteins (non-disulphide and disulphide cross-linked

TABLE 1. Effect of recombinant alpha A-interferon on the total amounts of extracted keratinocyte proteins

| | µg protein/fraction | | |
|---|---|---|---|
| Fraction | Control* | *r*α A-interferon (400 IU/ml) | Interferon as % of control |
| KG-macroaggregate ($R_2$) | 23·85 | 21·83 | 91·5 |
| KG-supernate ($S_2$) | 23·28 | 17·66 | 75.8 |
| SDS ($S_3$) | 50·63 | 42·03 | 83·0 |
| Non-disulphide cross-linked keratin ($S_4$) | 13·08 | 22·32 | 58·6 |
| Disulphide cross-linked keratin ($S_5$) | 96·96 | 119·01 | 122·7 |
| Envelope protein ($R_5$) | 29·16 | 31·73 | 108·8 |

Interferon was used here at a 400 IU/ml dose.
* Mean of duplicate determinations.

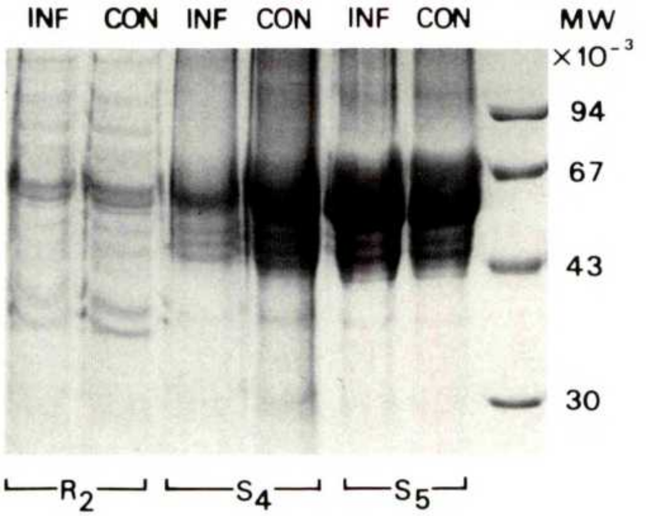

FIGURE 2. SDS-PAGE gel of keratohyalin granule associated proteins ($R_2$), of non-disulphide cross-linked proteins ($S_4$), and of disulphide cross-linked proteins ($S_5$) extracted from *r* IFN-αA treated keratinocyte cultures (IFN) and controls (CON). Right-hand lane contains molecular weight standards.

proteins). In this experiment, *r* IFN-α A increased the amount of disulphide cross-linked proteins while decreasing non-disulphide cross-linked proteins. However, the keratin bands showed no qualitative differences.

## DISCUSSION

In this study, we have demonstrated that recombinant alpha A-interferon markedly inhibited the *in vitro* DNA-synthesis of normal human keratinocytes at doses corresponding to physiological levels. [$^3$H]-TdR incorporation into DNA is a reliable measure of keratinocyte proliferation, especially if the radioactive label in the acid soluble fraction remains the same in treated cultures as in the controls. We did not observe any significant pool changes under interferon treatment, which exludes the possibility of an altered cellular uptake of [$^3$H]-TdR. Our results provide evidence that *r* IFN-α A exerts an anti-proliferative effect on normal human keratinocytes. These findings correspond well with those presented by Nickoloff *et al.* (1984) who investigated the influence of the same interferon type on keratinocyte proliferation by counting the keratinocytes before and after treatment.

Growth and proliferation of various cell types are inhibited by interference of *r* IFN-α A with different phases of the cell cycle (Clemens & MacNurlan, 1985). However, for human keratinocytes, the mechanism of interferon action at the molecular level has still to be elucidated. Yaar *et al.* (1984) suggested a mechanism involving surface receptors, since interferon action could be blocked by addition of choleragen.

The main advantage of human keratinocyte cultures as a model is their ability to synthesize a variety of keratins including the high-molecular weight keratins (Kragballe, Desjarlais & Marcelo, 1985), which are synthesized in terminally differentiated cells. Our results provide evidence that the stages of keratinocyte differentiation, as characterized by their keratin patterns, are altered by *r* IFN-α A. Most prominent is the accelerated formation of disulphide cross-linked keratins following addition of *r* IFN-α A. This may reflect a shift from lower to higher molecular weight cross-linked keratins and envelope proteins or an inhibition of the synthesis of lower-molecular weight keratins, or both. Thus, interferons may stimulate or support terminal differentiation in human keratinizing epithelia, whereas other drugs such as synthetic retinoids inhibit terminal differentiation *in vitro* (Stadler *et al.*, 1984). Furthermore, terminal differentiation is not directly correlated with the proliferative capacity of keratinocytes *in vitro*, suggesting that enzymes, for example transglutaminases for post translational protein modification may also be influenced. The significance of these findings for the possible clinical application of *r* IFN-α A remains to be elucidated.

## ACKNOWLEDGMENT

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# Precocious appearance of involucrin and epidermal transglutaminase during differentiation of psoriatic skin

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## SUMMARY

We compared the distribution in psoriatic skin of three different markers usually found in the stratum granulosum of normal skin. Using dansylcadaverine, we demonstrate that epidermal transglutaminase activity can be detected in most of the suprabasal layers of involved psoriatic skin and that the epidermal transglutaminase activity closely matches involucrin distribution. The glycoprotein GP37 was not detected in involved psoriatic skin of stable lesions. These results suggest that the integrated control of several independent pathways of terminal differentiation is lost in psoriasis, resulting in the classical feature of parakeratosis with absence of the stratum granulosum.

Epidermal transglutaminase is a soluble calcium- and sulphydryl-containing enzyme which mediates covalent cross-linking of polypeptide chains by $\varepsilon$-($\gamma$-glutamyl)lysine bonds (Peterson, Zettergen & Wuepper, 1983). In human epidermis, the major substrates of transglutaminase are involucrin (Watt, 1983) and keratolinin (Peterson & Weupper, 1984). These soluble precursor proteins are enzymatically cross-linked by transglutaminase into large polymers that form the insoluble cross-linked envelope of the keratinocyte. Buxman, Kocarnik and Holbrook (1979) have shown that transglutaminase is an important marker for terminal epidermal differentiation. Using dansylcadaverine to localize the epidermal transglutaminase, Buxman and Wuepper (1978) found that the distribution of fluorescence was restricted to the granular layer in normal epidermis and was markedly diminished or absent in psoriatic skin, and that the degree of diminution of fluorescence directly parallelled the degree of diminution of the granular layer. However, we recently showed that involucrin, which is normally detected in the granular layer of normal skin, was present in all but the basal layer of involved psoriatic skin (Bernard *et al.*, 1985). Since involucrin is a natural substrate for epidermal transglutaminase, we were led to re-

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examine the distribution of this enzyme in psoriatic skin. We have compared the distribution of involucrin and transglutaminase with that of a third marker, the glycoprotein GP37, which is also found in the granular layer of normal skin (Bonnefoy *et al.*, 1984).

## METHODS

### *Skin biopsies*

Biopsies were taken from the affected skin of 14 patients with psoriasis vulgaris by Dr P. Brun (CITP, Antibes, France). Recent lesions (present for less than 15 days) and stable lesions (present for more than one month) were studied. Skin biopsies from normal individuals with no history of psoriasis served as controls. The biopsies were quick-frozen in Tissue-Tec OCT compound (Lab-Tek Products Division, Napierville, IL, U.S.A.) using dry ice/acetone-isopentane and stored at −80°C until used.

### *Histochemical detection of transglutaminase*

Dansylcadaverine was used to detect transglutaminase activity (Buxman & Wuepper, 1978). This technique localizes the transglutaminase-catalysed binding of dansylcadaverine to the endogenous insoluble $\gamma$-glutamyl substrate. Frozen sections 5 $\mu$m thick were rapidly warmed to 37°C and immersed for 30 min at 37°C in 0·05 M Tris (hydroxymethyl) aminoethane with 0·15 M NaCl (TBS) containing 10 mM $CaCl_2$, 2 mM dansylcadaverine (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 2·5% dimethylsulphoxide (used to solubilize dansylcadaverine). Control substrate solutions lacked calcium or contained 10 mM ethylene diamine tetracetic acid (EDTA). After incubation, sections were washed at room temperature in two changes of buffer, and mounted in 70% glycerol in TBS. The sections were observed with a Zeiss epifluorescence microscope equipped with a BP 365 exciter filter and an FT510 reflector, and photographed using Kodak Tri-X film.

### *Immunofluorescence detection of involucrin and GP37*

Frozen sections 5 $\mu$m thick were stained using a 'sandwich technique'. After two washes in phosphate-buffered saline (PBS) to remove the tissue-tec OCT compound, slides were incubated for 30 min at room temperature with rabbit anti-involucrin anti-serum or guinea-pig anti-GP37 anti-serum, each diluted 1 : 100 in PBS containing 3% BSA (ELISA grade, Sigma).

After extensive washing with PBS, the slides were incubated for 30 min at room temperature with appropriate fluorescein-conjugated goat Ig fractions diluted 1 : 40 in PBS containing 3% BSA. After washing in PBS, the slides were mounted as above.

The anti-involucrin anti-serum was a generous gift from Dr R. Rice (Harvard School of Public Health, Boston, MA, U.S.A.). The fluorescein conjugates were purchased from Cappel Laboratories (Downingtown, PA, U.S.A.).

## RESULTS

### *Distribution of transglutaminase activity and involucrin*

When normal human epidermis was stained for transglutaminase activity using the dansylcadaverine method, fluorescence was detected mainly in the granular layer. The label was closely associated with the cell membrane (Fig. 1A). This staining was completely prevented if calcium was omitted or if 10 mM EDTA was added. The pattern of involucrin distribution was found to

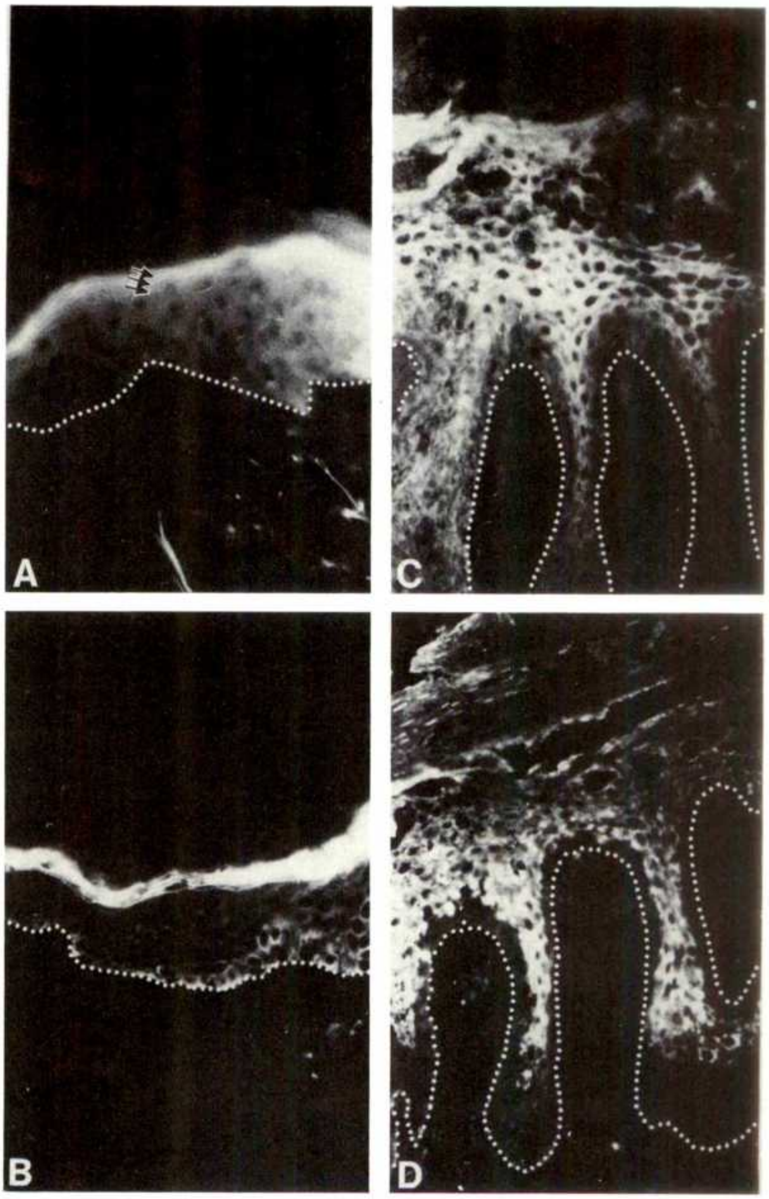

FIGURE 1. Histochemical localization of transglutaminase activity in normal skin (A), and involved psoriatic skin (C), and immunofluorescence staining of involucrin in normal skin (B), and involved psoriatic skin (D). The basement membrane is marked with a dotted line, In A, arrows indicate membrane associated-label. (Original × 200).

be very similar, being restricted generally to the three to four cell layers of the stratum granulosum (Fig. 1B).

However, when involved psoriatic epidermis was stained for transglutaminase activity, the fluorescence was detectable down to the suprabasal layer (Fig. 1C); staining was abolished by omission of calcium or addition of EDTA. The distribution of involucrin matched the

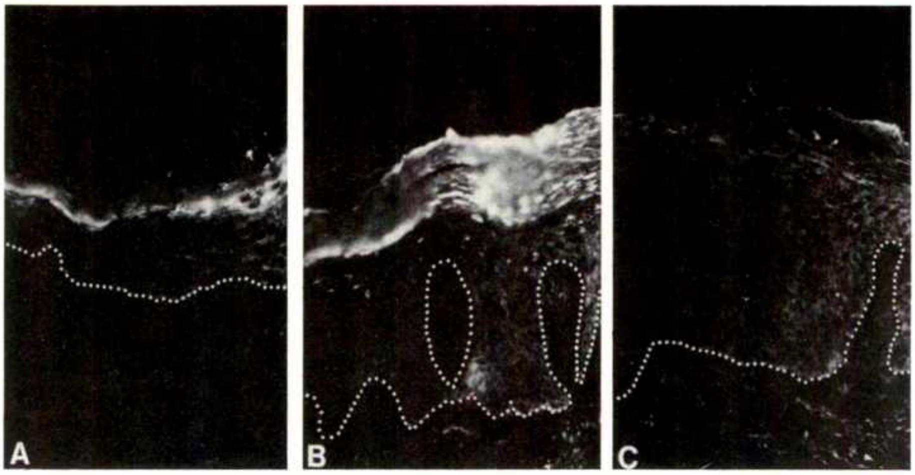

FIGURE 2. Immunofluorescence staining of GP37 in normal skin (A), and psoriatic skin from recent (B) and stable (C) lesions. The basment membrane is marked with a dotted line. (Original × 200).

transglutaminase activity (Fig. 1D). Similar results were obtained with all the 14 cases tested, irrespective of whether the lesions were recent or stable.

### *Distribution of GP37*

GP37 was detected as punctate cytoplasmic staining in the granular layer of normal skin (Fig. 2A) and non-involved psoriatic skin. However, when epidermis from 6 recent and 8 stable psoriatic lesions was stained for GP37, this compound was detected in the parakeratotic scales of the recent psoriatic plaques, as a punctate cytoplasmic staining of superficial flattened cells (Fig. 2B), but was totally absent in the stable lesions (Fig. 2C).

## DISCUSSION

We showed recently (Bernard *et al.*, 1985) that involucrin, which is normally detected in the stratum granulosum of normal skin, was detected in all but the basal layer of involved psoriatic skin. Together with the occurence of $\psi 3$ antigens in psoriatic skin (Mansbridge, Knapp & Strefling, 1984), the unexpected precocious appearance of involucrin suggested that the differentiation pathway of psoriatic keratinocytes was not simply truncated but fundamentally altered (Heenen & Galand, 1984).

Since involucrin is a natural substrate for epithelial transglutaminase (Peterson *et al.*, 1983, Thacher & Rice, 1985), we investigated the distribution of epithelial transglutaminase activity in psoriatic skin. Using the method of Buxman and Wuepper (1978), we found that not only was transglutaminase activity detectable, but that it matched involucrin distribution. Although Buxman and Wuepper did not find this histochemical distribution, our result fits in with their finding that buffered extracts of homogenized psoriatic scales contained significant amounts of transglutaminase activity in an *in vitro* enzyme assay (Buxman & Wuepper, 1978). Moreover, we showed that the reaction was specific since in all the 14 cases studied, the staining was totally abolished in the absence of $Ca^{++}$ and in the presence of EDTA. The distribution of GP37, although similar in normal skin to that of transglutaminase activity and involucrin, appears to be strikingly different in involved psoriatic skin. The results of GP37 staining of involved psoriatic skin suggest that GP37 synthesis is progressively reduced with time. Although still detectable in recent lesions, it is eliminated towards the upper layers of parakeratotic scales, and finally disappears in stable lesions.

The early appearance of involucrin and transglutaminase activity in psoriatic skin suggests that neither of these markers is specific for the granular layer since they were detected even in the absence of a granular layer. On the other hand, the disappearance of GP37 from stable psoriatic lesions argued for the granular layer specificity of this marker, since it correlates with the histological disappearance of the granular layer in psoriasis. We suggest that the terminal differentiation process of keratinocytes can be divided into several independent pathways: the onset of involucrin and transglutaminase activity, the expression of GP37, and the synthesis of specific acidic-basic pairs of keratins (Woodcock-Mitchell *et al.*, 1982). In psoriasis, the integrated control of these different pathways may be altered, some being favoured i.e. involucrin and transglutaminase, some being blocked, i.e. GP37, some being induced, i.e. $\psi$3 antigen (Mansbridge *et al.*, 1984) and some being qualitatively modified, i.e. keratins (Skerrow & Hunter, 1978). The classical histological change observed in chronic psoriasis, namely parakeratosis with an absence of the stratum granulosum, could thus be the result of the loss of this integrated control.

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# The effects of an aromatic retinoid (etretinate) on epidermal cell production and metabolism in normal and ichthyotic patients

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## SUMMARY

Normal subjects and ichthyotic patients were biopsied before and after etretinate (Tigason®) therapy. Histometric and cell kinetic measurements showed minimal changes following treatment. The metabolic activity across the epidermis was measured by a novel approach using the Quantimet 720 image analyser, and was significantly increased in the normal subjects as demonstrated by glucose-6-phosphate dehydrogenase (G6PDH), succinic dehydrogenase (SDH) and non-specific esterase (NSE) activities. In the ichthyotics studied the levels of G6PDH and SDH activity were significantly lowered following etretinate therapy, to levels similar to those demonstrated in normal subjects. In contrast, the activity of NSE was increased in the ichthyotics after etretinate therapy. It is suggested that the effect of retinoids is to 'normalize' the process of epidermal differentiation when it is abnormal.

The retinoids have a striking effect on epidermal differentiation, and it is probable that it is via this mechanism that they exert their therapeutic effects in psoriasis and disorders of keratinization. Their exact site of action is uncertain. Effects on glycoprotein synthesis, mannosyl transfer, lipid synthesis and cell replication have all been recorded, but which of these is fundamental to their action is uncertain (Marks *et al.*, 1984). Clearly these compounds have important biological effects that are only partially characterized in biochemical terms. The present study was carried out on normal and ichthyotic subjects, using cytochemical and cell kinetic techniques, to elucidate further the molecular basis of the clinical effectiveness of the retinoids.

## METHODS

### *Subjects*

The study received ethical approval from the local Joint Ethics Committee. Ten normal healthy

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male subjects, who had given their witnessed, written informed consent, (mean age 27 years, range 21–48 years) were given a total of 1 mg/kg/day of etretinate (Tigason®) in two orally administered doses daily for 28 days. Seven ichthyotic patients (four male, three female, mean age 27 years, range 5–55 years) who were about to undergo etretinate therapy, and who had given their informed consent, were also studied (Table 1).

TABLE 1. Details of the ichthyotic patients studied

| Type of ichthyosis | No. of patients |
|---|---|
| Autosomal dominant ichthyosis | 2 |
| Erythrokeratoderma variablis | 2 |
| Bullous ichthyosiform erythroderma | 1 |
| Non-bullous ichthyosiform erythroderma | 1 |
| Lamellar ichthyosis | 1 |

### *Biopsies*

The normal subjects were biopsied using a 4 mm disposable punch, before and after 4 weeks' treatment, and the patients were excision biopsied before and after 4–6 weeks' treatment. Each biopsy from the normal subjects, both pre- and post-treatment, was halved. One half of each biopsy was processed histologically for subsequent histometric analysis, and the other half was either quenched at −70°C for cytochemical assay or prepared for autoradiography. Thus a total of 10 specimens was obtained for histometric analysis both pre- and post-treatment, and five obtained for cytochemical assay and five for autoradiography both pre-and post-treatment.

Each biopsy from the seven patients, both pre-and post-treatment, was halved. One half of each biopsy was processed histologically for subsequent histometric analysis, and the other half quenched for cytochemical assay.

### *Histometric measurements*

Biopsies were fixed in 10% neutral formalin, routinely processed, and embedded in paraffin wax. Wax embedded tissue was sectioned at 5 μm and stained with haematoxylin and eosin (H&E). Measurements of mean epidermal thickness (MET), mean stratum corneum thickness (MSCT), mean total epidermal thickness (MTET) and the ratio of the lengths of the basal layer to the granular cell layer (B/G ratio) were made using the image editor and standard computer modules of the Quantimet 720 image analysing computer (Cambridge Instruments).

### *Thymidine autoradiographic labelling index determination*

Thin slices (1 mm) of skin from the normal subjects only were incubated in Eagle's MEM containing [$^3$H] thymidine (concentration 2·5 μCi/ml, specific activity 21 Ci/mmol) for 4 h at 37°C. Incubated skin was fixed in 10% formalin, routinely processed and embedded in paraffin wax. Wax-embedded tissue was sectioned at 5 μm and autoradiographs were prepared using Ilford K2 liquid emulsion. The sections were stained with H&E through the emulsion, and the number of labelled basal and suprabasal cells were counted (minimum of 1000 counted) and expressed as a percentage of the total number of basal cells. This was designated the labelling index (LI).

### *Cytochemical assays for enzyme activities*

Biopsies were quenched in hexane cooled to $-70°C$ by an alcohol-solid $CO_2$ mixture, stored in liquid nitrogen until sectioning, and sectioned at 10 $\mu m$ on a Bright's motor-driven cryostat, prior to estimation of glucose-6-phosphate dehydrogenase (G6PDH), succinic dehydrogenase (SDH), and non-specific esterase (NSE) activities, using previously established techniques (Chayen, Bitensky & Butcher, 1973; Pearse & Marks, 1978). The section thickness and quality of the preparations were carefully monitored and the whole process was standardized for all specimens examined (Pearse & Marks, 1974). The density of the reaction products was measured, using the image editor and densitometer modules of the Quantimet 720 in a novel manner as described below. Pre-and post-treatment biopsies were assayed at the same time.

The enzyme density profile was measured using a 10 × 10 pixel scanning frame. The frame was moved stepwise across the epidermis so that each successive frame position was directly adjacent to the previous position (Fig. 1). There were 24 frame steps in each scan line ($x_1$–$x_{24}$).

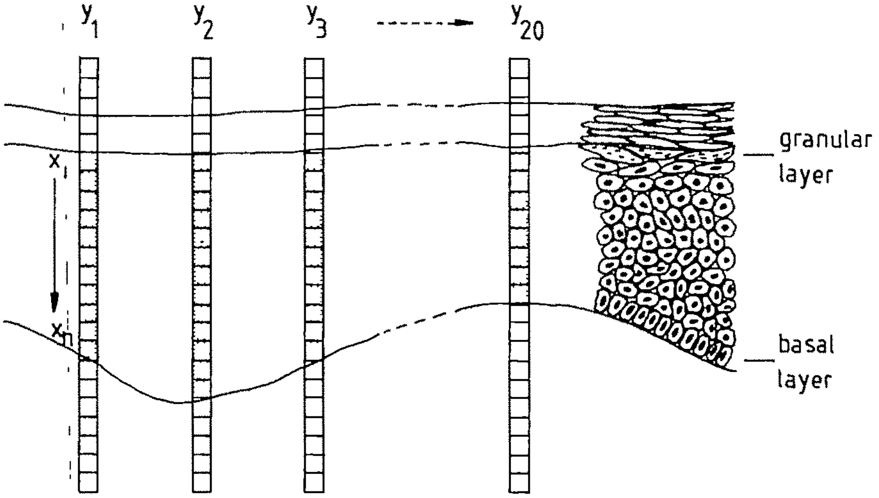

FIGURE 1. Diagram representing the method by which the mean density profile across the epidermis was calculated. $y_1$–$y_{20}$ represents the number of scan lines across the epidermis and $x_1$–$x_n$ the frames used in each scan line.

Then the starting point of the frame scan was moved 50 pixels along the epidermis and the scan repeated. This procedure was repeated 10 times per section in two consecutive sections (scan lines $y_1$–$y_{20}$), giving a total of 480 frame positions. Only the data from those areas that lay within the epidermis (selected using the image editor) were used. The number of readings used from each scan line ($x_1$–$x_n$) was therefore proportional to the thickness of the epidermis. To calculate a mean distribution of enzyme density across the epidermis, the results from each scan line were arranged in 10 arbitrary frames or 'bins', where the enzyme reaction product density for any given scan frame ($x_i$, $y_j$) was placed into an arbitrary frame or bin, numbered 1–10, corresponding to its relative position across the epidermis, where:

$$\text{Bin number} = \text{the integer of}\left(\frac{10(i-1)+5}{n}\right)+1$$

where $i$ is the number of the scan frame and $n$ is the total number of scan frames within the epidermis in the particular scan line.

For example, if there were 16 readings within the epidermis in a particular scan line, the consecutive readings would be placed respectively into bins 1, 1, 2, 3, 3, 4, 5, 5, 6, 6, 7, 8, 8, 9, 10, 10. When the readings from all 20 scan lines had been allocated to their respective bins, the total reaction product density in each bin was divided by the number of readings in that bin to give a mean density for that level of the epidermis.

### *Statistics*

A two-way analysis of variance was used to evaluate the cytochemical data and a one way analysis of variance to evaluate the histometric data.

## RESULTS

### *Normal Subjects*

#### *Histometric measurements*

The results of the histometric measurements of MET, MSCT, MTET and B/G ratios are shown in Table 2. There was no statistically significant difference in any of the histometric parameters measured before and after 28 days' etretinate therapy.

TABLE 2. Mean epidermal thickness (MET), mean stratum corneum thickness (MSCT), mean total epidermal thickness (MTET) and basal/granular cell ratio (B/G) in 10 normal subjects before and after etretinate therapy. (Figures are means ± SD)

| Etretinate treatment | MET (μm) | MSCT (μm) | MTET (μm) | B/G ratio |
|---|---|---|---|---|
| Before | 60·1 ± 1·1 | 18·9 ± 1·4 | 79·2 ± 2·4 | 1·11 ± 0·07 |
| After | 60·1 ± 1·9 | 18·2 ± 0·7 | 78·4 ± 2·5 | 1·14 ± 0·05 |

#### *Labelling index determinations*

The mean labelling index (± SD) was 4·9 ± 1·2% before etretinate treatment and 6·8 ± 4·2% after treatment. Although the labelling index was raised following treatment, this difference was not statistically significant.

#### *Cytochemical assays*

*Succinic dehydrogenase*. The SDH activity in normal subjects before and after etretinate treatment is shown in Figure 2. There was a consistent increase in the density of the reaction product and therefore in enzyme activity throughout all levels of the epidermis after etretinate therapy. These differences were statistically significant ($P < 0{\cdot}01$).

*Glucose-6-phosphate dehydrogenase*. Although the distribution of this reaction product differed from that due to SDH activity, there was also an increase in the reaction product due to G6PDH activity throughout the epidermis following etretinate treatment (Fig. 3). These differences were statistically significant ($P < 0{\cdot}01$).

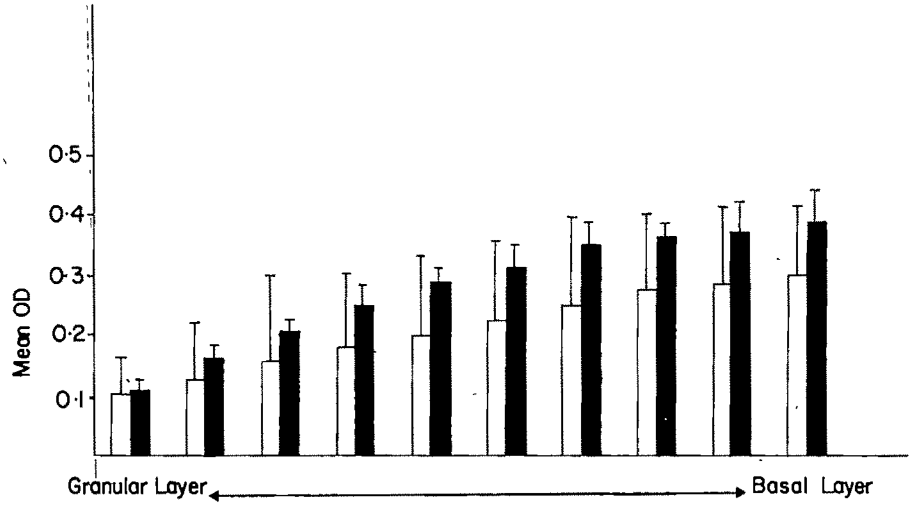

FIGURE 2. Succinic dehydrogenase (SDH) activity across the epidermis of normal subjects before (☐) and after (■) etretinate treatment. Each column represents the mean of five subjects (± SD).

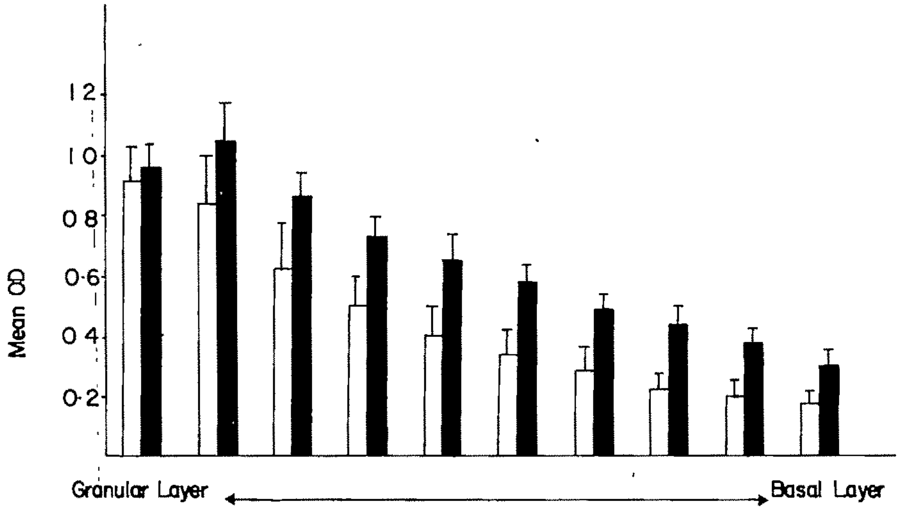

FIGURE 3. Glucose-6-phosphate dehydrogenase (G6PDH) activity across the epidermis of normal subjects before (☐) and after (■) etretinate treatment. Each column represents the mean of five subjects (± SD).

*Non specific esterase.* Deposition of the reaction product due to NSE activity is normally found exclusively in the granular cell layer. It was found to be significantly increased following treatment ($P < 0·01$) (Fig. 4).

*Ichthyotic patients*

*Mean epidermal thickness*
The epidermal thickness (mean ± SD) was 120·6 ± 47·1 μm before treatment and 132·0 ± 71·1 μm after treatment. This difference was not statistically significant.

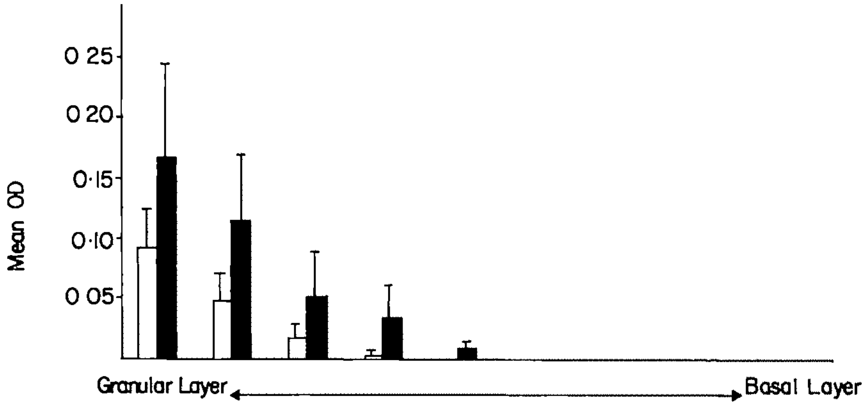

FIGURE 4. Non specific esterase (NSE) activity across the epidermis of normal subjects before (□) and after (■) etretinate treatment. Each column represents the mean of five subjects (± SD).

*Cytochemical assays*

*Succinic dehydrogenase.* It can be seen from Figure 5 that there was a decrease in the deposition of the reaction product due to SDH activity following treatment. These differences were statistically significant ($P < 0.01$). The levels in ichthyotic patients after etretinate treatment were very similar to those obtained in normal subjects before treatment.

*Glucose-6-phosphate dehydrogenase.* The deposition of the reaction product due to G6PDH activity was decreased in all patients following treatment. These differences were statistically significant ($P < 0.01$). Figure 6 shows that following etretinate therapy the level of G6PDH

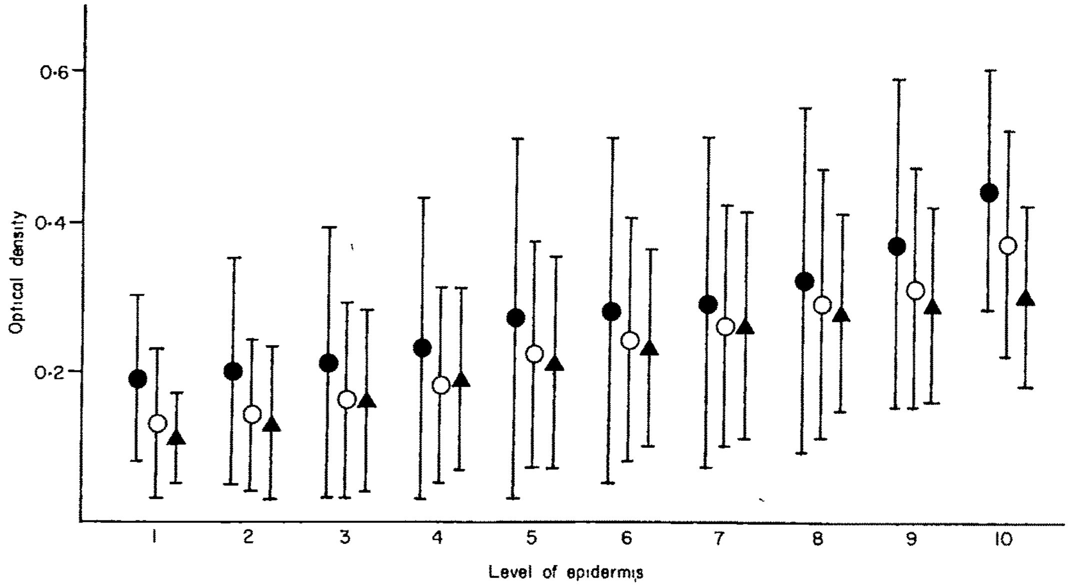

FIGURE 5. Succinic dehydrogenase (SDH) activity across the epidermis of ichthyotic subjects before (●) and after (○) etretinate treatment compared with that in normal subjects (▲). Points represent mean values of seven ichthyotic patients or five normal subjects (± SD).

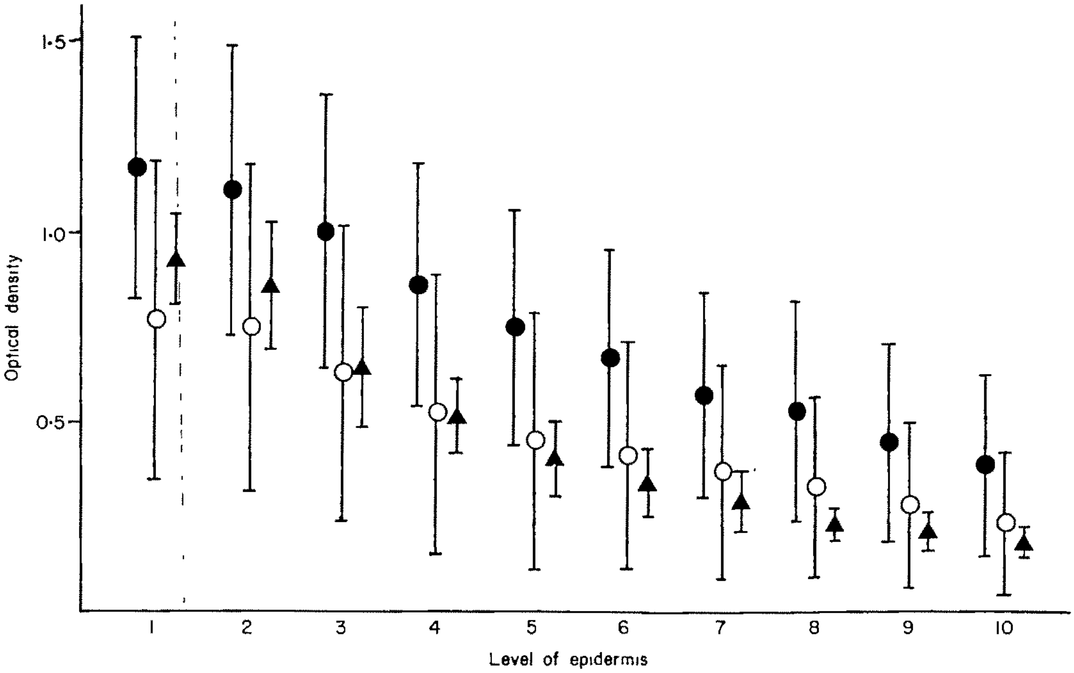

FIGURE 6. Glucose-6-phosphate dehydrogenase (G6PDH) activity across the epidermis of ichthyotic subjects before (●) and after (○) etretinate treatment, compared with the values obtained for normal subjects (▲). Points represent the mean values of seven ichthyotic patients or five normal subjects (± SD).

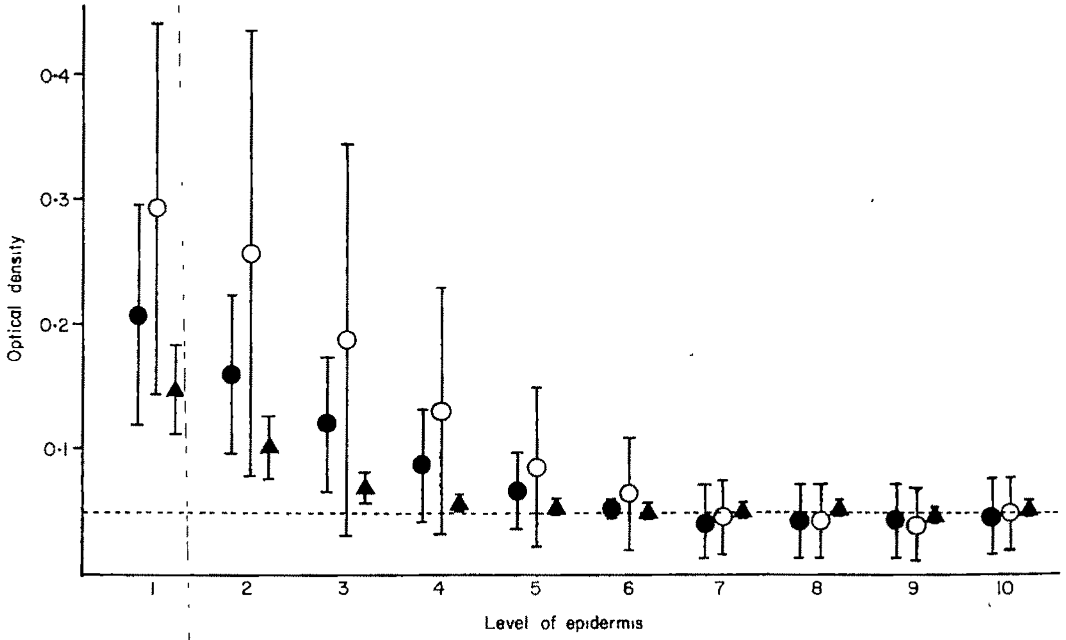

FIGURE 7. Non-specific esterase (NSE) activity across the epidermis of ichthyotic subjects before (●) and after (○) etretinate treatment, compared with the values obtained for normal subjects (▲). Points represent the mean values of seven ichthyotic patients or five normal subjects (± SD). The post treatment levels were significantly increased in levels 1–5 of the epidermis in the ichthyotics (two way analysis of variance). The dotted line represents the background level.

activity throughout the epidermis was very similar to that seen in normal subjects before treatment.

*Non-specific esterase.* The densities of the reaction products indicated that the activity of this enzyme was increased in the upper layers of the epidermis following treatment (Fig. 7). These differences were statistically significant ($P < 0{\cdot}01$). Both the pre- and post-treatment levels of NSE activity were higher than those seen in normal untreated subjects.

## DISCUSSION

There have been many reports of the clinical effectiveness of the retinoid group of drugs in the treatment of the ichthyotic disorders, psoriasis, acne and in malignant disease of the epidermis (*see* reviews by Ward *et al.*, 1983; Ward *et al.*, 1984; Marks, 1983; Orfanos *et al.*, 1985). Tsambos and Orfanos (1981) described changes in keratinocytes as well as in psoriatic dermis, following oral retinoid therapy. Various ultrastructural and histochemical modifications to the psoriatic epidermis after retinoid treatment have also been described, including the accumulation of mucosubstances (Jablonska *et al.*, 1981) and vacuolation of keratinocytes and the deposition of granular material intercellularly. There have also been numerous reports describing biochemical changes in the epidermis after retinoid administration, including marked changes in glycoprotein synthesis (De Luca, 1977; King & Tabiowo, 1981) and alterations in the transfer of sugar residues via a retinol linked sugar (mannosyl-retinyl phosphate) (Wolf *et al.*, 1979).

However, there have been relatively few studies which have investigated the effects of retinoids on normal skin. Tsambos, Mahrle & Orfanos (1980) described marked epidermal changes in guinea-pig epidermis after high dosages of etretinate. Hairless mice fed aromatic retinoid over a 21-day period showed cell kinetic and histometric changes in the epidermis (Fritsch *et al.*, 1981). These included an increase in the [$^3$H] thymidine labelling index due to a shortening of the S phase, and an increase in epidermal thickness which was most apparent after 14 days of treatment.

In the present study no statistically significant changes were found in the thickness of the epidermis or the stratum corneum following 28 days of treatment. Significant epidermal thickening has, however, been noted in our previous studies using Roaccutane (Marks *et al.*, 1981; Kingston, Gaskell & Marks, 1983). The differences between these results probably reflect differences in the effects of retinoids in the different groups of patients investigated. There were no changes in cell proliferation as measured by thymidine labelling index in either the normal subjects or the ichthyotic patients after treatment with etretinate. This observation is in agreement with our previous findings (Marks *et al.*, 1981) but not with those of Orfanos (1982), who found small increases in the labelling index and shortening of the cell cycle after the earlier times of 7 and 14 days treatment with etretinate in normal subjects.

The cytochemical assays demonstrated that retinoid treatment significantly increased the activity of certain energy generating metabolic pathways throughout the whole epidermis in normal individuals. This was true of the Krebs cycle marker SDH, the marker for the pentose shunt pathway, G6PDH and also the marker for lysosomal hydrolytic enzyme activity NSE. Conversely, the raised levels of activity of G6PDH and SDH found in the ichthyotic patients prior to treatment were reduced after treatment to levels similar to those in normal individuals. Lysosomal NSE activity, however, was further increased in the ichthyotic patients after treatment.

Changes have been observed in other enzyme systems following retinoid treatment. For example, Lowe, Kaplan and Breeding (1982) found a reduction in ornithine decarboxylase (ODC) activity following treatment of psoriatic patients with etretinate. However, in hairless mice Connor and Lowe (1983) found that the retinoid treatment induced ODC activity, and concluded that the induced ODC activity was found only after the retinoids had been depleted. The ability to both inhibit and to induce ODC activity may be related to the action of retinoids as weak tumour promoters under certain circumstances and as inhibitors of promotion under others. This polyamine biosynthetic enzyme has been shown to increase in proliferating cell or tissue systems (Raima *et al.*, 1970; Tabor & Tabor, 1976).

Retinoids have a wide variety of actions on epidermis and it is difficult to know whether an observed effect is itself fundamental or secondary to another, earlier, alteration. From previous observations it seems unlikely that the changes described here are the result of a direct action on the enzyme activities investigated. It is of considerable interest that the changes in the normal subjects and in the patients were in opposite directions, with the levels of activity in the patients reverting towards 'normal' after treatment. This probably indicates no more than that the effect of the retinoid is to 'normalize' the process of epidermal differentiation when it is abnormal. We believe that these findings help to further characterize the effects of retinoids on the epidermis and emphasize their action on the process of epidermal differentiation.

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# Proteolytic enzymes in blister fluids from patients with dermatitis herpetiformis

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## SUMMARY

Proteolytic enzymes may be involved in blister formation in dermatitis herpetiformis (DH). We have examined collagenase, gelatinase and elastase-like enzyme activities in fluids collected from spontaneous blisters and from suction blisters raised on developing DH-lesions induced by application of potassium iodide. Control suction blisters were raised on unaffected DH-skin and on healthy volunteers. High enzyme activities were found in spontaneous blisters, and suction blister fluids obtained from developing lesions showed increased levels of gelatinase and elastase-like enzymes. Inhibitor studies revealed that a part of the elastase-like enzyme activity might be derived from inflammatory cells. Gel filtration chromatography disclosed two separate elastase-like enzymes which, as they had high molecular weights, could be either enzyme-inhibitor complexes or aggregates.

Dermatitis herpetiformis (DH) is a blistering skin disease with an underlying gluten-sensitive enteropathy (Katz, 1980). The rash responds well to sulphone drugs, and treatment with a strict gluten-free diet leads to remission of the skin lesions (Fry *et al.*, 1982). However, the mechanism of blister formation is unknown although the presence of pathognomic IgA and complement deposits in the papillary dermis, with characteristic polymorphonuclear leukocyte accumulation in developing blisters, suggest complement and leukocyte mediated inflammatory reactions (Haffenden *et al.*, 1980; Reitamo *et al.*, 1981). Recently, we have demonstrated the presence of collagenase and elastase activities in blister fluids derived from various bullous skin diseases (Oikarinen *et al.*, 1983). Highest enzyme activities were found in samples collected from patients with DH, suggesting that proteolytic enzymes might be directly involved in blister formation in this condition.

In the present study we have examined further the role of proteolytic enzymes in the pathogenesis of DH. Firstly, we have measured collagenase, gelatinase and elastase activities in suction blister fluids obtained from potassium iodide (KI) induced lesions and from unaffected

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skin of DH patients. Secondly, we have compared several synthetic substrates in order to determine whether different types of enzymes are involved. Finally, we have analysed the blister fluids by gel filtration chromatography to examine the elastase-like enzymes in more detail.

## METHODS

### *Blister fluids*

Seven DH patients were examined. All patients had active disease and had used dapsone (50–100 mg/day) to control the blistering. Dapsone was stopped three days before collecting the blister fluids. KI was applied to the skin as described previously (Reitamo *et al.*, 1981). Briefly, 50% KI in petrolatum was applied to unaffected abdominal skin using aluminum cups (Finn chamber®). The cups were removed after 24 h, the lesions were observed, and suction blisters were induced exactly on the KI application sites as described previously (Kiistala, 1968). Control suction blisters were induced on contralateral abdominal skin after application of petrolatum only. Control suction blister fluids were obtained from the abdominal skin of eight healthy, age-matched volunteers. The volume of blister fluid varied from 0·5 ml to 2·0 ml and the samples were stored at −20°C until assayed.

Blister fluids were also collected from spontaneous blisters on two of the patients and the cells in these blister fluids were immediately separated by cytocentrifugation. The cell pellets and the supernatants were stored at −20°C. The cell pellets were homogenized in a glass-glass homogenizer in 50 mM Tris-HCl, pH 7·8 at 4°C, and the homogenates were centrifuged at 15 000 ***g*** for 20 min. The supernatants were removed and used for enzyme assays.

### *Enzyme assays*

For the collagenase assay, blister fluid samples were incubated with $^3$H-labelled type I collagen (Ryhänen *et al.*, 1982) as described previously (Oikarinen *et al.*, 1983).

Gelatinase activity was assayed using a synthetic substrate 2,4-dinitrophenyl-L-prolyl-L-glutaminyl-L-glycyl-L-isoleucyl-L-alanyl-L-glycyl-L-glutaminyl-D-arginine(DNP-peptide; Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) (Masui *et al.*, 1977). After incubation with blister fluid samples in 50 mM Tris-HCl, pH 7·6, containing 0·2 M NaCl and 5 mM $CaCl_2$, the digestion of the substrate was determined by the addition of 2·5 volumes of 1 M HCl, followed by extraction of the amino-terminal degradation fragment containing the DNP-group into ethyl acetate. The absorbance of the organic layer was measured at 365 nm.

Elastase-like enzyme activity was assayed by using the synthetic substrate, succinyl-(L-alanyl)3-paranitro-anilide (SAPNA; Sigma Chemical Co., St. Louis, MO, U.S.A.) (Bieth, Spiess & Wermuth, 1974) as described previously (Oikarinen *et al.*, 1983).

The hydrolytic activity of the blister fluids was also assayed using a synthetic substrate alanyl-paranitroanil-ide (ala-PNA; Vega Biochemicals, Tucson, AZ, U.S.A.) under similar conditions to those used for SAPNA (Ito, Honda & Mori, 1982). The reaction was monitored by the change in absorbance at 410 nm, and hydrolytic activity expressed as μmol ala-PNA hydrolysed/h/ml of blister fluid.

In the inhibitor studies, blister fluid samples were incubated with phenylmethylsulphonyl fluoride (PMSF), a serine protease inhibitor, before measurement of elastase-like enzyme activity.

### *Gel filtration*

Blister fluid samples 0·5 ml were chromatographed on a Sepharose CL-4B (Pharmacia Inc., Piscataway, NJ, U.S.A.) column, 2·5 × 90 cm, equilibrated and eluted with 1 M NaCl, 5 mM CaCl$_2$, and 50 mM Tris-HCl, pH 7·6 at 4°C. Two ml fractions were collected and aliquots were used for enzyme assays. The protein concentration was measured by absorbance at 280 nm. The void volume (V$_0$) of the column was determined using Dextran Blue 2000 (Pharmacia), and the total volume (V$_t$) was estimated from the elution of $^3$H$_2$O (Amersham). The column was calibrated with thyroglobulin (m.w. 669 000), ferritin (m.w. 440 000), catalase (m.w. 232 000) and aldolase (m.w. 158 000) (all from Pharmacia).

The α2-macroglobulin concentration in the blister fluids was determined by radial immunodiffusion (Mancine, Carbonara & Heremans, 1965), using 0·6% agarose gels containing 25 mM Tris-HCl, pH 7·4, and 112 ng/ml rabbit anti-human α2-macroglobulin (Calbiochem-Behring).

## RESULTS

### *Enzyme activities*

A high level of collagenase was detected in spontaneous blisters in DH patients (data not shown). In contrast, no measurable collagenase activity was found in suction blister fluids obtained from the unaffected or KI induced lesional skin of DH patients, or those from the healthy controls.

Fluids from spontaneous blisters and suction blisters induced on affected skin in DH patients showed gelatinase activity using DNP-peptide as substrate (Table 1). No gelatinase activity was detected in blister fluids from unaffected skin of DH patients or from healthy controls.

Low levels of elastase-like enzyme activity, using SAPNA as substrate, were found in the suction blister fluids from unaffected skin of DH patients, the activity being similar to that in blister fluids from the controls (Fig. 1a). KI application significantly increased the enzyme activity ($P < 0{\cdot}025$, Students *t*-test), but the activity was still markedly less than the activity in

TABLE 1. Activities of DNP-peptide, SAPNA and ala-PNA hydrolysing enzymes in fluids from spontaneous and suction-induced blisters in dermatitis herpetiformis patients

| | Mean enzyme activity/ml original blister fluid | | |
|---|---|---|---|
| Sample | DNP-peptide hydrolysis $\Delta A_{365}$/h/ml | SAPNA hydrolysis nmol/h/ml | ala-PNA hydrolysis μmol/h/ml |
| Spontaneous blister | | | |
| Supernatant | 0·76 (2)* | 90·7 (2) | 0·90 (2) |
| Cell pellet | 0·22 (2) | 60·7 (2) | 0·11 (2) |
| Suction blister | | | |
| Potassium iodide-treated skin | 0·94 (1)** | 7·3 (5) | 0·62 (4) |
| Unaffected skin | Not detectable (5) | 3·3 (5) | 0·54 (3) |

* Figures in parentheses indicate numbers of blister fluids tested.

** Affected but not potassium iodide treated skin in this patient.

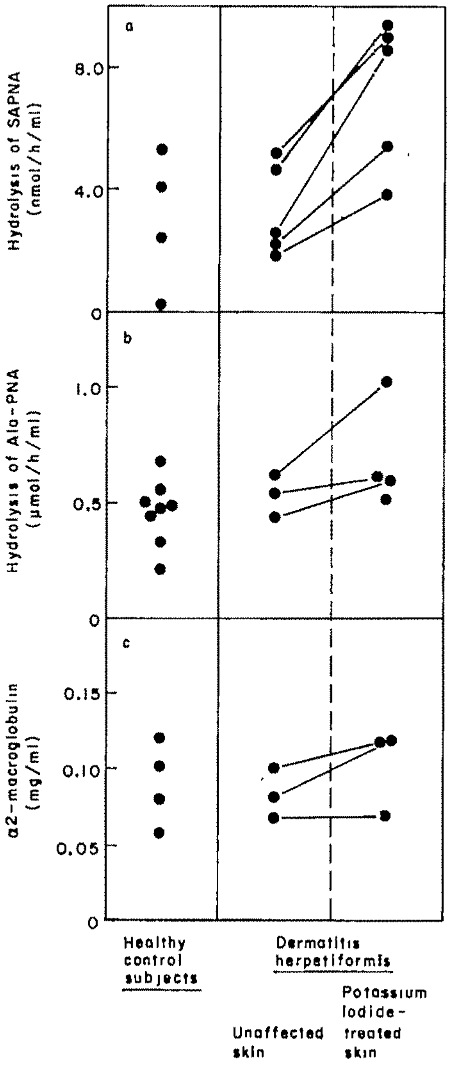

FIGURE 1. Elastase-like enzyme activities and α2-macroglobulin concentrations in suction blister fluids from patients with dermatitis herpetiformis and healthy controls: (a) hydrolysis of SAPNA; (b) hydrolysis of ala-PNA; (c) α2-macroglobulin concentration. Each point is the mean of duplicate assays and solid lines connect values from the same patient.

fluids from spontaneous blisters in DH patients (Table 1). In KI-induced skin lesions, elastase activity in suction blister fluids did not differ between two patients with macroscopic vesicles and three patients without vesicles. In the controls, serum elastase-like enzyme activity was higher than that in the suction blister fluids from the patients; mean activity (± SD) was 8·1 ± 6·0 nmol SAPNA hydrolysed/h/ml of serum.

Levels of hydrolytic activity for ala-PNA were found to be similar in suction blister fluids from control subjects and from the unaffected and KI-induced lesional skin of DH patients

(Fig. 1b). The levels were not markedly different from those in spontaneous blisters in DH patients (Table 1). Ala-PNA hydrolysing activity in the serum of the control subjects was slightly higher than that in blister fluids; mean activity (± SD) 1·6 ± 0·4 μmol/h/ml of serum.

To study the balance between proteolytic enzymes and their inhibitors, α2-macroglobulin concentrations were measured in suction blister fluids (Fig. 1c). The mean levels (± SD) were 0·10 ± 0·02 mg/ml in the controls, 0·09 ± 0·02 mg/ml in the unaffected skin of DH patients, and 0·10 ± 0·03 mg/ml in KI-induced skin lesions. These levels were about 10% of the corresponding serum levels. The ala-PNA hydrolysing activity in suction blister fluids from KI-induced skin lesions was not inhibited by PMSF. In contrast, 2 mM PMSF caused a 30% inhibition of SAPNA hydrolysing activity in these blister fluids.

### *Gel filtration*

The supernatants from spontaneous blisters in DH patients gave three major protein peaks on gel filtration (Fig. 2a). The first peak eluted close to the void volume of the column $(V_0)$, the second large peak between fractions 40 and 70, and the third peak close to the total volume of the column $(V_t)$. Similarly, three protein peaks were found in the cell pellet, but the total amount of protein was considerably less than in the supernatant (Fig. 2b). The SAPNA-hydrolysing enzyme activity in the supernatant eluted in two major peaks (Fig. 2a). The first peak (I) eluted close to the void volume, and the second peak (II) between fractions 38 and 50. The highest activity was noted in the fractions corresponding to a molecular weight of approximately 240 000. In cell pellets, the SAPNA-hydrolysing activity also eluted in two peaks (Fig. 2b), their positions corresponding approximately to those in the supernatant. The ratio of enzyme activity in the two peaks (I : II) was 0·11 in the supernatant and 0·20 in the cell pellet.

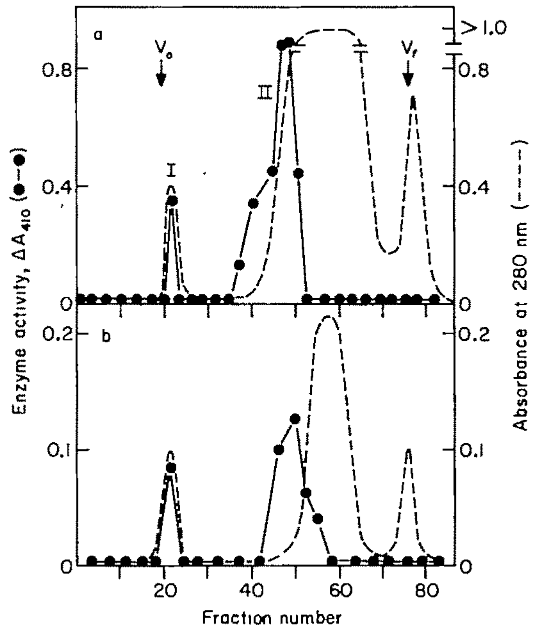

FIGURE 2. Elution profile of SAPNA hydrolysing enzyme activity on gel filtration chromatography of (a) the supernatant and (b) the cell pellet, from spontaneous blisters in DH patients.

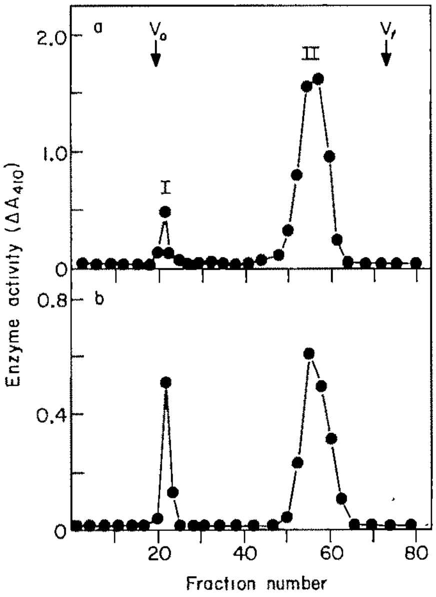

FIGURE 3. Elution profile of ala-PNA hydrolysing enzyme activity on gel filtration chromatography of (a) the supernatant and (b) the cell pellet from spontaneous blisters in DH patients.

Ala-PNA hydrolysing enzyme activity also eluted in two major peaks both in the supernatant and cell pellet samples (Fig. 3a, b). The first peak (I) eluted close to the void volume and the second (II) between fractions 50 and 64. Peak enzyme activity was found in fraction 55 which corresponded to a molecular weight of approximately 170 000. The ratio of enzyme activity in the two peaks (I:II) was 0·09 in the supernatant and 0·34 in the cell pellet.

## DISCUSSION

We found that fluids from suction blisters induced on unaffected skin of patients with DH had low levels of elastase-like enzyme activity, levels which differed very little from those in suction blister fluids from control subjects. No collagenase or gelatinase activity was detected in these blisters. In contrast, high levels of collagenase, gelatinase and elastase activity were found in spontaneous blisters, indicating that all these enzymes are released in mature DH-lesions, perhaps from polymorphonuclear leukocytes (Haffenden *et al.*, 1980; Reitamo *et al.*, 1981). The reason for the absence of collagenase activity in suction blister fluids from KI-induced DH-lesions remains to be elucidated. The presence of $\alpha 2$-macroglobulin, an inhibitor of a variety of proteolytic enzymes, including collagenases, could explain this (Travis & Salvesen, 1983).

A significantly increased elastase-like enzyme activity was found in suction blister fluids from developing DH-lesions. The increase in SAPNA hydrolysing activity seemed specific since the levels of $\alpha 2$-macroglobulin and ala-PNA hydrolysing enzyme activity did not show this change. Although KI application produces, in DH patients, skin lesions which are clinically and histologically identical with spontaneous lesions, and polymorphonuclear leukocytes appear in these lesions after 6–24 h (Haffenden *et al.*, 1980; Reitamo *et al.*, 1981), the pathogenic significance of increased elastase-like enzyme activity in developing and mature DH-lesions remains unclear. However, it is known that pathognomonic IgA complexes are located in close association with the microfibrillar part of the elastic fibres, and that these complexes tend to disappear during blister formation in DH, perhaps as a result of proteolytic degradation of elastic fibres (Yaoita, 1978; Reitamo *et al.*, 1981; Egelrud & Bäck, 1984). In contrast to SAPNA, ala-PNA hydrolysing enzyme activity was not increased in DH-blister fluids. Since the cell pellets from spontaneous DH-blisters contained very low levels of ala-PNA hydrolysing activity and the enzyme activities in the supernatants were the same as in the control sera, it appears that this enzyme is derived from serum. The low levels of ala-PNA hydrolysing activity found in suction blister fluids from the controls would support this.

Gel filtration showed that most of the SAPNA and the ala-PNA hydrolysing activity eluted in different fractions, indicating that two separate enzymes are responsible. The ala-PNA hydrolysing enzyme differs from leukocyte and pancreatic elastases which readily use SAPNA as substrate, but do not hydrolyse ala-PNA (A. Oikarinen, unpublished observation). Moreover, the ala-PNA hydrolysing activity was not affected by PMSF, a serine protease inhibitor which affects both leukocyte and pancreatic elastases and which we found to cause a 30% inhibition in SAPNA hydrolysing activity. The molecular weight determinations for SAPNA and ala-PNA hydrolysing enzymes gave values of 240 000 and 170 000, respectively. These values are considerably higher than the molecular weights previously reported for elastases, including the leukocyte elastase (Bieth, 1978). Whether the elastase-like enzymes found in the present study occurred in aggregates or enzyme-inhibitor complexes is unknown. Further studies are required, especially to elucidate the role of the SAPNA hydrolysing enzyme in blister formation in DH.

## ACKNOWLEDGMENTS

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# Eczematous reactions in atopic patients caused by epicutaneous testing with inhalant allergens

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## SUMMARY

To determine whether inhalant allergens could induce eczematous lesions we studied 17 patients with atopic eczema (with or without allergic rhinitis), 13 patients with allergic rhinitis without atopic eczema and 10 healthy control subjects. The allergens, birch pollen (*Betula verrucosa*) and house dust mite (*Dermatophagoides pteronyssinus*), were applied in aluminium chambers for 48 h on clinically normal skin. In 17 patients with atopic eczema, six epicutaneous test reactions of the delayed type to birch pollen and three to house dust mite were seen at 48 or 72 h. In 13 patients with allergic rhinitis without eczema there was one delayed reaction to birch pollen and none to house dust mite. No delayed type test reactions to either allergen were seen in the controls. Biopsies of the positive test sites revealed an eczematous reaction with epidermal spongiosis and microvesiculation. Immunostaining of cryostat sections showed dermal cell infiltrates consisting of mainly T lymphocytes (ratio of T4:T8, 2–6:1) and to a lesser degree Langerhans and indeterminate T6+ cells. 50–90% of the cells were Ia+. The numbers of basophils and mast cells did not exceed 10–15%.

The eczematous lesions in atopic dermatitis show cellular characteristics similar to a delayed type hypersensitivity reaction, with the T lymphocyte as the predominant cell type in the inflammatory infiltrates of the skin (Leung *et al*., 1983; Zachary, Allen & MacDonald, 1985). A large number of patients with atopic dermatitis show skin reactions of the immediate type when tested with various inhalant or food allergens, whereas there have been only a few reports of responses of the delayed type (Go, Michaels & Ellis, 1970; Platts-Mills *et al*., 1983). It has proved difficult to show that these allergens could cause the patients' eczema, which is a delayed type response. Only in infants does there seem to be a clear correlation between food allergy and atopic dermatitis (Sampson & Albergo, 1984). Although hyposensitization with various pollen preparations has proved to be effective in atopic states such as allergic rhinitis it has not been effective in the treatment of atopic eczema. However, it has been shown recently that allergen

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extracts or purified antigen applied as a patch test to mildly abraded skin of patients with atopic dermatitis can induce an eczematous skin reaction (Mitchell *et al.*, 1982; Platts-Mills *et al.*, 1983). This has been taken as evidence that inhalant allergens could be aetiological agents in atopic dermatitis also. Skin biopsies of such patch test reactions have shown a dermal inflammatory cell infiltrate consisting of mononuclear cells, neutrophils, basophils and eosinophils. The large number of basophils suggests that these reactions represent cutaneous basophil hypersensitivity rather than classical delayed hypersensitivity (Dvorak, 1976; Askenase, 1979; Mitchell *et al.*, 1984).

To study the skin response to inhalant allergens we used two allergens which are known to cause positive skin reactions of the immediate type in a large number of atopic patients in Finland: birch pollen and house dust mite. Birch pollen was chosen because of the clinical observation that some patients show symptoms of both rhinitis and eczema during the pollen season.

## METHODS

### *Patients with atopic eczema*

Seventeen patients (11 men, 6 women) with diagnosed atopic eczema (mean age 24·8 years; range 5–42 years) were studied when in remission. All had suffered from moderate to severe skin inflammation which involved the sites typical of atopic eczema. Nine of these patients had seasonal allergic rhinitis and three had bronchial asthma. One patient had urticaria due to cow's milk. Thirteen patients had a positive prick test reaction to birch pollen, and nine patients to house dust mite. In six patients with a positive prick test to birch pollen the RAST test was performed and showed specific IgE to birch pollen in all six. Four patients showed a clear exacerbation of their eczema during the birch pollen season. Testing during the pollen season in May was avoided.

### *Control subjects*

One control group consisted of 13 atopic patients (9 men, 4 women; mean age 29·2 years, range 17–44 years), with seasonal allergic rhinitis but without any history of atopic eczema. Eleven of these patients had a positive prick test reaction to birch pollen. Another control group consisted of 10 healthy subjects (4 men and 6 women, mean age 32·7 years, range 24–48 years), with no signs of atopy.

### *Allergens*

Commercial lyophilized preparations of Aquagen SQ 108 birch pollen (*Betula verrucosa*) and Aquagen SQ 503 dust mite (*Dermatophagoides pteronyssinus*) were purchased from Allergologisk Laboratorium A/S, Copenhagen, Denmark. Distilled water and white petrolatum were used as vehicles. Both allergens were used at 500-fold concentration as compared to the prick test standardized to 1 HEP (histamine equivalent prick).

### *Epicutaneous testing and skin biopsies*

Epicutaneous testing was performed with aluminium Finn Chambers (Epitest Ltd., Helsinki, Finland) on clinically normal skin on the back. The chambers were removed after 48 h. In the first seven patients the test reactions were read at 48 h only. In the remaining patients and all the controls the reactions were read at 48, 72 and, in some cases, also at 96 h. As controls of the epicutaneous testing procedure, chambers were applied empty and containing vehicle only.

When a positive epicutaneous test reaction was seen, biopsies were taken from the positive reaction site and the control site. In three patients with a positive epicutaneous test reaction a biopsy was taken at 48 and 72 h.

### *Processing of skin biopsies*

The biopsies were immediately divided into two parts. One was snap-frozen in liquid isopentane at −50°C and serial cryostat sections of 5 $\mu$m were fixed in acetone for 5 min for immunoperoxidase staining of cell surface antigens. The other half was further divided into two parts; one fixed in Bouin's fluid (Hénocq & Gaillard, 1981) and the other in Karnovsky's fixative (Dvorak *et al.*, 1974) for the enumeration of mast cells and basophils.

### *Immunostaining*

The following primary antibodies were used: OKT3, OKT4, OKT6, OKT8, OKT9, OKM1, OKIa1 (all from Ortho Diagnostic Systems Inc., Raritan, NJ, U.S.A.), Leu 7 (Becton-Dickinson, CA, U.S.A.) and anti B cell antibody (Dakopatts A/S, Copenhagen, Denmark). The immunoperoxidase staining was performed with the avidin-biotin-complex (ABC) method (Vectastain ABC kit mouse IgG PK-4002, Vector Laboratories Inc., Burlingame, CA, U.S.A.) as described by Hsu, Raine and Fanger (1981). The sections were incubated in darkness with 0·5% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., MO, U.S.A.) and 0·01% $H_2O_2$ in phosphate-buffered saline (PBS), counterstained with haematoxylin, dehydrated in a graded alcohol series, cleared in xylene and mounted in Histoclad (Clay Adams, NJ, U.S.A.).

The following controls of the immunostaining were performed: (1) the first antiserum was replaced by PBS or a nonrelevant monoclonal antibody, and (2) tissue sections were stained for endogenous peroxidase activity without antiserum treatment.

### *Mast cells and basophilic granulocytes*

Mast cells and basophils were stained from paraffin sections with toluidine blue and with Giemsa's stain.

### *Cell counts*

Inflammatory cell subclasses were counted in tissue sections at × 400 magnification. At least 200 inflammatory cells were counted in each specimen by two independent investigators.

TABLE 1. Incidence of positive skin test reactions to birch pollen (*Betula verrucosa*)

| | | Type of skin test reaction positive | | | |
|---|---|---|---|---|---|
| Clinical diagnosis | Number of subjects | Immediate and delayed | Immediate only | Delayed only | No reaction |
| Atopic eczema | 17 | 6 | 7 | 0 | 4 |
| Allergic rhinitis without eczema | 13 | 1 | 10 | 0 | 2 |
| Healthy controls | 10 | 0 | 0 | 0 | 10 |

## RESULTS

### *Patch test reactions*

Positive patch test reactions to birch pollen or house dust mite produced erythematous and vesicular lesions at the test site. Compared with other patch test reactions of the allergic type, e.g. nickel, the reactions were usually less indurated. The results of the epicutaneous testing with birch pollen are shown in Table 1. In the first seven of the 17 patients with eczema, in whom the test reactions were read at 48 h only, there were no positive delayed reactions to house dust mite and only one patient showed a positive reaction to birch pollen. In the remaining 10 patients whose test reactions were read also at 72 h, one showed a folliculitis type reaction to the aqueous and petrolatum controls as well as to pollen. This patient's test reaction, therefore, was not considered positive. In the other nine patients, five showed a positive reaction to birch pollen; three at 48 h and all five at 72 h. In five of the six patients with a positive epicutaneous test, a RAST test was positive for birch pollen; in the remaining patient no RAST test was performed. In three patients, positive test reactions to birch pollen and house dust mite could be elicited both with aqueous and with petrolatum preparations. Of the three patients with a positive patch test to house dust mite, two had a positive prick test to house dust mite, and all three also had a positive delayed reaction to birch pollen.

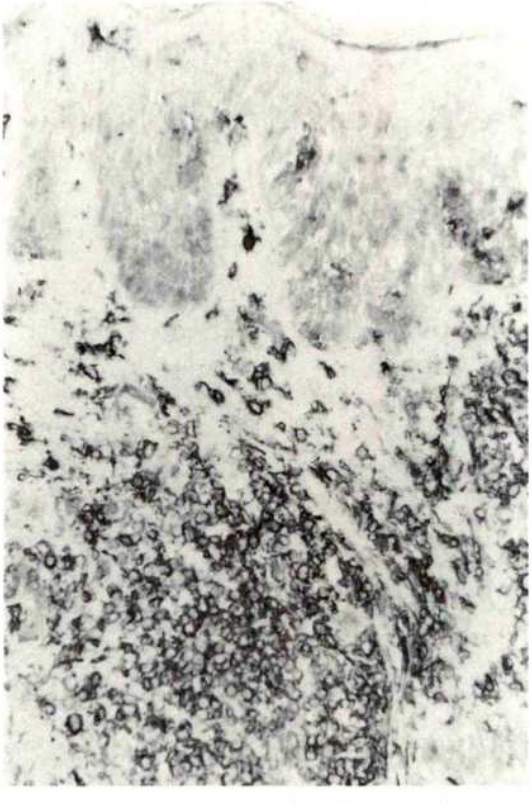

FIGURE 1. Biopsy of a positive patch test reaction to birch pollen at 72 h. Immunoperoxidase staining with OKIa1 shows that most inflammatory cells in the dermis are Ia-positive. (Original × 400).

In the patient with atopic dermatitis and cow's milk allergy there was a positive epicutaneous reaction to both birch pollen and cow's milk. The patient developed a weal and flare at the site of the milk patch test during the first hour of testing. Mild but typical atopic eczema developed in his cubital and popliteal areas, wrists and face at the time of testing.

In the group of atopic patients with allergic rhinitis but without eczema, one positive epicutaneous reaction to birch pollen occurred in a patient who also gave a positive prick test reaction to birch pollen. No positive reactions to house dust mite were seen.

No positive delayed type reactions to birch pollen or to house dust mite were seen in the healthy controls.

### *Immunohistology*

In all patients with a positive epicutaneous test reaction, the biopsy from the test site showed an eczematous skin reaction with epidermal spongiosis and vesiculation. Immunostaining of frozen sections with monoclonal antibodies showed dermal cell infiltrates consisting mainly of T lymphocytes. The T4:T8 ratio was 2–6:1. 50–90% of the infiltrating cells were Ia-positive (Fig. 1). There was a smaller proportion (0–30%) of T6-positive cells (Langerhans cells and indeterminate cells) in the dermal infiltrate. In some small infiltrates almost all the cells were T6+ (Fig. 2). In the epidermis the number of T6+ cells exceeded the number of Ia+ cells. Occasional T8+ cells could be seen in the epidermis but no T4+ cells were observed.

### *Mast cells and basophilic granulocytes*

The number of mast cells and basophils in the dermal infiltrates in positive test reactions to both birch pollen and house dust mite was the same in toluidine blue and Giemsa stained sections. There was no difference in the numbers of these cells seen between tissue fixed in Bouin's fluid and that fixed in Karnovsky's fixative. The number of the mast cells and basophils was usually 5–10% and never exceeded 15%.

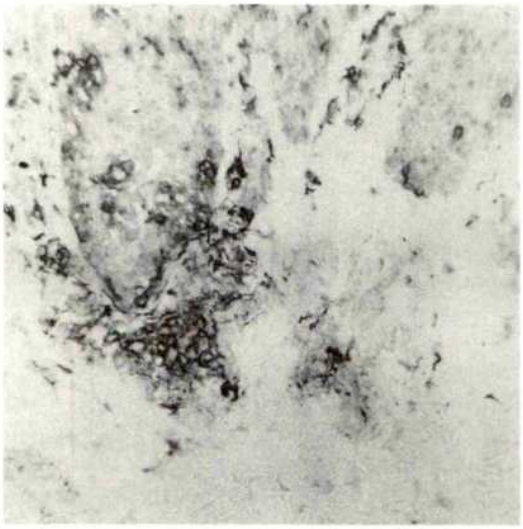

FIGURE 2. Positive patch test reaction to birch pollen from the same patient as in Figure 1. Immunoperoxidase staining with OKT6 shows that most cells of the dermal infiltrate are T6-positive (Original × 250).

## DISCUSSION

The present results indicate that it is possible to produce a delayed type reaction in the skin of some atopic patients by epicutaneous testing with inhalant allergens. The use of very high concentrations of the allergen could provoke an eczematous reaction of the irritant type. More highly purified antigens might allow the use of lower concentrations of the antigen. At present it is not possible to distinguish clearly an allergic from an irritant reaction by studying the cellular infiltrates (Reitamo *et al.*, 1981; Scheynius *et al.*, 1984). The positive test reactions developed slowly, 48–72 h after the application of the test substance. This militates against the possibility of an irritant reaction. Use of vehicle controls excluded false positive reactions. Only one patient gave a positive reaction to the vehicle.

Our study confirms the recent finding with house dust mite antigen P1, that it is possible to produce a delayed type response both *in vivo* and in cell culture, to purified P1 antigen (Mitchell *et al.*, 1984; Rawle, Mitchell & Platts-Mills, 1984). A similar time-course for the patch test reaction was seen in these studies (Mitchell *et al.*, 1982). In the cellular infiltrate of the acute eczematous lesions these authors identified basophils, eosinophils, mononuclear cells and neutrophils. In the present study we did not observe extensive mast cell or basophil infiltration in the dermis as these cells never exceeded 15% of the total infiltrate. One reason for the low numbers of basophils might be that we did not use the optimal technique for the identification of basophils, i.e. embedding in methacrylate. However, as the total number of basophils and mast cells observed was so low, it seems unlikely that there were large numbers of basophils in the dermal infiltrates. Dvorak *et al.* (1974) have shown that basophils appear in varying numbers in classical delayed type reactions in man. It has been postulated that only weak antigens can elicit cutaneous basophil hypersensitivity reactions, and that when there is antibody formation the response changes to the delayed type (Mahapatro & Mahapatro, 1984). In the natural lesions of atopic eczema, the predominant cell type is a T lymphocyte which is Ia+ and T4+ (Leung *et al.*, 1983; Zachary *et al.*, 1985) and our results are in accordance with this.

A T6 positive cell population of Langerhans and indeterminate cells was seen in the dermis. Earlier studies have shown T6+ cells in normal dermis (Murphy *et al.*, 1983) and increased numbers of T6+ cells in the allergic patch test reactions (Ralfkiaer & Wantzin, 1984; Berti *et al.*, 1985) and other delayed-type responses (Konttinen *et al.*, 1983) including natural lesions in atopic eczema (Leung *et al.*, 1983; Zachary *et al.*, 1985).

We have found a group of patients who have a birch pollen allergy of the immediate type and who also show a seasonal exacerbation of their atopic eczema in spring during the birch pollen season. The present study included four such patients, three of whom showed a positive delayed type response to birch pollen. The results would therefore indicate that some inhalant allergens, e.g. birch pollen and possibly house dust mite, could be causative factors in atopic eczema.

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# Cold urticaria and virus infections: a clinical and serological study in 39 patients

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## SUMMARY

Thirty-nine patients with cold urticaria seen over a 12-year-period were re-examined. All but 12 still had positive skin tests for cold and only five of these had shown a spontaneous cure. Fourteen patients were prone to collapse on cold exposure. The incidence of atopy in this group was comparable to that in control groups. Cold urticaria is an extremely chronic disease. The mean disease duration was 9·3 years.

Serum antibodies to Epstein-Barr virus, measles virus, cytomegalovirus (CMV), varicella-zoster virus (VZV), herpes simplex virus (HSV), *Chlamydia psittaci* and *Mycoplasma pneumoniae* were determined in all 39 patients and compared with control groups. The EBV-antibody patterns (heterophile antibodies and different types of EBV-specific antibodies) showed no evidence of current or of recent primary or secondary infection with EBV. Complement fixing antibody titres to measles virus, CMV, HSV and *Mycoplasma pneumoniae* were significantly higher in cold urticaria patients than in controls.

The existence of a basic immuno-regulatory defect responsible for both the cold urticaria and the elevated antibody levels is proposed.

Virus infections, especially infectious mononucleosis (Africk & Halprin, 1969; Cowdrey & Reynolds, 1969; Wands, Perrotto & Isselbacher, 1976) and hepatitis (Vaida, Goldman & Bloch, 1983) have been implicated in the aetiology of urticaria for many years. There have also been isolated reports of possible associations with cytomegalovirus (Humphreys, 1975), measles virus vaccination (Zwemer *et al.*, 1971) and ornithosis (Weber & Stetter, 1969).

Cold urticaria, in particular, has been described in infectious mononucleosis (Barth, 1981; Tyson & Czarny, 1981; Lemanske & Bush, 1982; Bonnetblanc *et al.*, 1983; Wu, 1983), hepatitis (Clarke, 1969) and measles (Kobacker & Parkhurst, 1935). In most cases the urticaria accompanied the onset of the infection and disappeared spontaneously after the infection subsided. In some studies the urticaria was chronic (Weber & Stetter, 1969; Zwemer *et al.*, 1971; Vaida, Goldman & Bloch, 1983). In four of the seven published cases of cold urticaria and infectious mononucleosis, the symptoms could be explained by the presence of cryoglobulins in

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the serum. There has also been evidence suggesting that virus infections might be related to the onset of asthma (Frick, 1983) or atopic dermatitis (Rystedt, Strannegard & Strannegard, 1984).

We postulated that inadequate handling of virus infections by the immunological apparatus might be the cause of chronic cold urticaria. We therefore re-examined a group of cold urticaria patients who had been seen over the past 12 years, for the presence of serum antibodies, in particular to the herpes viruses, but also to hepatitis B, measles virus, *Chlamydia psittaci* and *Mycoplasma pneumoniae*.

## METHODS

### *Patients*

Patients who had been seen at the urticaria out-patient clinic since 1971 took part in the study. The patients had been routinely studied according to a protocol which included a questionnaire, tests for physical urticaria, atopy, infections and oral provocation tests for aspirin and food additives (Doeglas & Bleumink, 1975); most of them had been seen at regular intervals over several years.

For the present study the patients were re-examined during the autumn of 1982. The skin tests for cold urticaria were repeated and blood was drawn for virus serology. Patients with combined cold contact- and cholinergic-heat urticaria were excluded.

### *Tests for cold urticaria*

All patients had been positive to one of the following tests at their first examination:

1. Cooling of a 3 cm diameter area of skin surface on the upper arm, using a device which kept the skin temperature at 0–4°C, for 10 min. If this test was negative:
2. Exposure of one lower arm, under venous occlusion, to a bath at 12°C, for 10 min. If negative, the test was repeated with both arms. If this was negative:
3. Exposure of the patient, in light clothes, in a room at 4°C, for 10 min.
4. For patients with a history of cold urticaria during excercise only: bicycling on an ergometer to produce a pulse rate of 140/min for 10 min, performed in a room at 4°C.

### *Laboratory tests*

The following tests were repeated: serum cryoglobulins with immunological determination of the immunoglobulin type, cold agglutinins, biphasic cold haemolysins, total serum IgG, IgA, IgM and IgE, total eosinophils, antinuclear antibodies and RAST IgE antibodies against house dust, house dust mite, cat, dog, grasses, moulds, milk and eggs.

### *Assessment of atopy*

Evidence for an atopic constitution was evaluated on the basis of four sets of criteria: a personal or first degree family history of asthma, hay fever or atopic dermatitis; the results of intracutaneous tests or RAST tests with a battery of foods and inhalant antigens (at least two reactions postive); the total serum IgE values (over 100 IU/ml); and the total eosinophil count in the peripheral blood (over 300/mm$^3$). These parameters were evaluated separately, but a patient was arbitrarily considered to be an atopic if at least three of these characteristics were present (modified from Doeglas, 1975).

### *Serology*

Antibodies to measles virus, cytomegalovirus (CMV), varicella-zoster-virus (VZV), herpes simplex virus (HSV), *Chlamydia psittaci* and *Mycoplasma pneumoniae* were determined using the micromodification of the complement fixation method (Casey, 1965). Heterophile antibodies were tested using the Paul-Bunnell Davidsohn method (Davidsohn & Lee, 1964). This reaction was considered to be positive at titres of 32 upwards.

Epstein-Barr virus (EBV) specific antibodies, specific for the virus capside antigen (VCA), (Henle & Henle, 1966), the early antigen (EA (D+R)) (Henle, Henle & Klein, 1971) and the EBV nuclear antigen (EBNA) Reedman & Klein, 1973) were measured by immunofluorescence methods.

Tests for IgM antibodies to EB-VCA were performed after precipitation of IgG from the sera with an antiserum specific for human IgG (Gispen *et al.*, 1975). HBV markers were detected with a solid phase radioimmunoassay (Abbott Laboratories, U.S.A.).

Thirty-nine control values for each antibody titre were chosen at random from data obtained in the routine laboratory during the same period of time. Data from patients with a known history or symptoms of cold urticaria or with a laboratory confirmed or seriously suspected acute infection with one of the micro-organisms tested, were excluded.

### *Statistical methods*

The Wilcoxon rank sum test was performed for comparison of antibody titres.

## RESULTS

Of 52 consecutive patients with proven cold urticaria seen over the previous 12 years, 39 were re-examined. There were 14 men and 25 women, aged from 10–71 years (median 36 and mean 37 years). The duration of symptoms at the time of re-examination was from 4 months to 35 years (median 5, mean 9·3 years). Re-examination took place from 4 months to 13 years after the original diagnosis of cold urticaria. Of the 39 patients, at the original examination 36 had typical cold contact urticaria. Three patients were classified as 'cholinergic cold urticaria' (Czarnetzki, Frosch & Sprekeler, 1981; Illig, Paul & Brück, 1980; Petres & Kunick, 1978). They responded with urticaria to a cold room but sometimes needed additional exercise in the cold to develop symptoms. All three showed the large erythematous macules and tiny weals as seen in cholinergic heat urticaria.

The results of skin tests for cold at the time of the first visit and at the follow up visit are shown in Table 1. In general there was a slight decrease in severity of symptoms over the years and this was confirmed by the tests. Generally, larger skin surfaces had to be exposed to the cold to obtain a positive result at follow up than at the first visit, e.g. an arm instead of an area of 3 cm diameter, or both arms instead of one. In 12 of the 39 patients all tests had become negative. In five of these this was interpreted as a spontaneous cure. However, the remaining seven still reported skin lesions on cold exposure, notwithstanding the negative tests.

Systemic symptoms after cold exposure were reported by 20 patients; these consisted of collapse in 14 patients, an erythematous flush in six, nausea and vomiting in six, headache in four and dyspnoea in three. A combination of cold urticaria with another type of physical urticaria was demonstrated in three patients, two also had factitious urticaria (dermographism) and one had delayed and persistent dermographism. Three patients with cold urticaria also had spontaneous idiopathic urticaria.

There was a personal history of atopic conditions in eight patients and a family history in 13. Intracutaneous tests with atopic allergens were positive in six patients (two of these were also RAST positive). There was a total serum IgE value of over 100 IU/ml in 10 patients and over

200 IU/ml in seven of these. The total eosinophil count in the peripheral blood was over 300/mm$^3$ in five patients. One patient scored in all four of these categories and three patients in three. These four patients were considered atopic according to our definition.

Cryoglobulin determinations had been performed repeatedly in all patients. Cryoglobulins were detected in four patients, but on only one of the three to six occasions on which determinations had been carried out in each patient. The cryoglobulins were of the IgM type in two patients, IgG in one and a mixture of IgG and IgA in one. There was no relation between the presence of cryoglobulins and the intensity of the clinical symptoms of cold urticaria. No cold agglutinins or biphasic cold haemolysins were demonstrated in any of the 39 patients.

Total serum immunoglobulins were determined on repeated occasions; a marked and persistently lowered level of IgA was present in four patients. One of these patients showed an IgG paraprotein with lambda II side chain specificity. Cryoglobulins were absent and his C1 esterase inhibitor level was normal. Only one patient showed antinuclear antibodies (titre 1:1000, homogeneous pattern) without anti dsDNA antibodies. There were no other immunological disturbances in this patient during the follow up period of 13 years.

The clinical history of virus infections in our patients was not unusual. There was a history of hepatitis in four patients, of infectious mononucleosis in two and severe recurring herpes labialis in one patient. One patient reported the onset of cold urticaria after a smallpox vaccination.

Serological tests specific for primary EBV infections (Paul-Bunnell Davidsohn test and EB-VCA IgM test) were negative in all 39 patients. The median IgG antibody titre for EB-VCA was slightly higher in the cold urticaria patients than in controls but the difference was not statistically significant (Table 2). Antibodies to EBNA showed no significant difference between patients and controls (Table 2). Of the 39 patients, five had low levels of antibodies to EBV-EA (titre 1:20) which is similar to those in healthy controls (Favart *et al*, 1978; Henle, Henle & Horwitz, 1979). The complement fixing antibody titres to measles, CMV, HSV, VZV, *Chlamydia psittaci* and *Mycoplasma pneumoniae* are shown in Table 3. Median antibody titres were significantly higher in the patients than in the controls for measles ($P=0.001$), CMV ($P<0.05$), HSV ($P<0.05$) and *Mycoplasma pneumoniae* ($P=0.01$), but not for VZV or *Chlamydia psittaci*. High titres of antibodies to VZV ($\geq 1:64$) were found in eight of the patients' sera but in none of the control sera.

In many cold urticaria patients there were high titres for complement fixing antibodies to more than one of the six organisms tested. Twenty-four patients had titres of $\geq 1:64$; 10 patients

TABLE 1. Skin tests for cold urticaria

| | | No. of patients positive | |
|---|---|---|---|
| Temperature | Test site | First visit | Follow up |
| 0–4°C machine | upper arm skin area 3 cm diameter | 28 | 17 |
| 12°C water | one lower arm | 6 | 5 |
| 12°C water | both lower arms | 1 | 2 |
| 4°C room | whole body | 3 | 2 |
| 4°C room + exercise | whole body | 1 | 1 |
| Total | | 39 | 27 |

TABLE 2. Antibody titres to EB-VCA and EBNA in cold urticaria patients and controls

| | Number of subjects | | | |
|---|---|---|---|---|
| | EB-VCA IgG | | EBNA | |
| Antibody titre | patients | controls | patients | controls |
| < 1:20 | | | 7 | 9 |
| < 1·40 | 2 | 1 | | |
| 1·20 | | | | 3 |
| 1:40 | 2 | 1 | 10 | 6 |
| 1.80 | | | 9 | 10 |
| 1:160 | 12 | 21 | 6 | 8 |
| 1:320 | | | 4 | |
| 1:640 | 20 | 10 | 3 | 3 |
| 1·1280 | 1 | 2 | | |
| 1:2560 | 1 | 4 | | |
| 1:10240 | 1 | | | |
| Median titre | 1.640 | 1:160* | 1:80 | 1·80* |

* No statistically significant difference between patients and controls.

TABLE 3. Antibody titres to measles virus, CMV, VZV, HSV, *Chlamydia psittaci* and *Mycoplasma pneumoniae* in cold urticaria patients and controls

| | | No. of subjects with antibody titre | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Antigen | Subjects | < 1:8 | 1:8 | 1:16 | 1:32 | 1:64 | ≥ 1:128 | Median titre | *P* |
| Measles virus | patients | 13 | 2 | 3 | 13 | 4 | 4 | 1:32 | 0·001 |
| | controls | 18 | 9 | 11 | 1 | 0 | 0 | 1:8 | |
| CMV | patients | 13 | 2 | 2 | 8 | 8 | 6 | 1:32 | < 0·05 |
| | controls | 23 | 2 | 2 | 3 | 5 | 4 | < 1:8 | |
| VZV | patients | 17 | 7 | 2 | 4 | 1 | 8 | 1:8 | NS |
| | controls | 15 | 18 | 3 | 3 | 0 | 0 | 1:8 | |
| HSV | patients | 13 | 2 | 3 | 7 | 6 | 8 | 1:32 | < 0·05 |
| | controls | 15 | 6 | 8 | 7 | 2 | 1 | 1:8 | |
| *Chlamydia psittaci* | patients | 30 | 4 | 3 | 2 | 0 | 0 | < 1:8 | NS |
| | controls | 31 | 5 | 2 | 0 | 1 | 0 | < 1:8 | |
| *Mycoplasma pneumoniae* | patients | 18 | 7 | 6 | 4 | 2 | 2 | 1:8 | 0·01 |
| | controls | 26 | 11 | 1 | 1 | 0 | 0 | < 1:8 | |

to two organisms, six to three organisms and one to four organisms. High titres to measles, *Mycoplasma pneumoniae* and VZV were always accompanied by high titres to one, two or three other organisms. This was also the case with high CMV and HSV titres in most but not all sera.

Comparison of the antibody titres of the 12 patients with negative skin tests with the 27 patients with positive skin tests showed no appreciable differences. The antibody titres in the four atopic patients and the six patients with positive intracutaneous tests to atopic allergens were similar to the antibody titres in the other patients. However, with these small groups statistical comparisons were not feasible.

Only three patients showed antibodies to hepatitis B surface antigen (HBs) and no HBs antigen was detected.

## DISCUSSION

Cold urticaria is a strikingly chronic disease, a fact which is insufficiently appreciated due to a lack of follow up studies. Some patients only appear cured as a result of their ingenuity in avoiding cold exposure. The negative skin tests in some patients with persistent subjective symptoms are probably explained by the variability of the symptoms over a period of time, which is seen in many types of physical urticaria. Systemic symptoms are frequent and the tendency to collapse endangers the life of these patients.

When symptoms are severe, an ice cube test over a small skin surface is usually positive. We found that when disease severity decreases it is sometimes necessary to increase the skin surface that is cooled in order to obtain a positive test result, e.g. an arm instead of an area of 3 cm diameter, or both arms instead of one. This has already been suggested by Illig and Kunick (1969).

The possible existence of a 'cholinergic cold urticaria' has been debated under various names (Petres & Kunick, 1978; Illig *et al.*, 1980; Czarnetzki, Frosch & Sprekeler, 1981). In our experience, some patients only develop symptoms on exposure to both cold and excercise, showing skin symptoms identical to those of cholinergic (reflex heat) urticaria. The term generalized cold urticaria seems less appropriate because less severe forms of cold contact urticaria may need generalized cold exposure to develop symptoms, without the need for excercise, and without the typical 'cholinergic' skin symptoms.

Cryoglobulins appeared to be present in our patients incidentally and not to play a role in the symptomatology. In general there were no coherent immunological abnormalities.

Atopy was not over represented in our cold urticaria patients. Four patients with multiple indications of atopy in a group of 39 is comparable to earlier findings in patients with cold urticaria compared to controls (Doeglas, 1975). There did not seem to be a relation between the presence of atopy or positive intracutaneous tests and the levels of antibodies. This is of interest in view of the findings of Rystedt, Strannegard & Strannegard (1984) who found increased EBV titres in patients with atopic dermatitis.

Several authors have reported the occurrence of cold urticaria of recent onset in infectious mononucleosis (IM) patients. In most of the studies, laboratory confirmation of the diagnosis of IM was based on haematological tests and the presence of heterophile antibodies and not on the presence of a typical EBV- specific antibody pattern. No data on the prevalence of active EBV-infections among cold urticaria patients have been published to our knowledge. The present study provides no evidence of an important relationship between EBV and cold urticaria of longer duration.

It has been established that heterophile antibodies are present in sera of 80–90% of adult IM patients (Henle, Henle & Horwitz, 1979). IgM antibodies to EBV-VCA are found in all IM patients and antibodies to EBV-EA in about 80% (Henle, Henle & Horwitz, 1979). During the acute primary infection there are no antibodies to EBNA present, whereas levels of IgG antibodies to EB-VCA are usually already high at the time of examination (Henle, Henle &

Horwitz, 1979). None of our cold urticaria patients showed this complete EBV-specific antibody pattern which is typical for acute primary IM. During the convalescent phase, heterophile antibodies and IgM antibodies to EB-VCA are negative, IgG to EBV-VCA is still elevated, antibodies to EBV-EA are decreasing and antibodies to EBNA appear but are still low in titre (Henle, Henle & Horwitz, 1979). Re-infection with or re-activation of EBV is usually reflected by elevated levels of antibodies to EBV-EA, EBNA and EV-VCA (IgG) and absence of IgM antibodies to EB-VCA and of heterophile antibodies. Normal healthy immune persons usually possess low to moderate levels of antibodies to EBNA and IgG antibodies to EB-VCA but no heterophile antibodies or IgM antibodies to EB-VCA (Henle, Henle & Lennette, 1979). Occasionally, healthy immune individuals have low antibody titres (⩽1:20) to EBV-EA (Favart *et al.*, 1978; Henle, Henle & Horwitz, 1979). The EBV-specific antibody patterns found in our cold urticaria patients and also in the control group closely resembled the normal pattern. A similar finding of normal EBV-antibody levels (IgG to VCA) in chronic urticaria was reported recently by Rystedt *et al.* (1984) in a study in which they showed increased EBV-antibody titres in patients with atopic dermatitis. In the latter patient group antibody titres to VZV and HSV were normal. The incidence of HBs antibodies was similar to that in control groups in our laboratory.

Surprisingly, the titres of antibodies to many of the other organisms tested for in our study were markedly elevated. These included antibodies to measles virus, CMV, HSV, and *Mycoplasma pneumoniae*. In addition, the cold urticaria patients had markedly higher percentages of relatively high titres of antibodies to VZV and to VCA-IgG.

The failure to find any difference between patients and controls in titres of antibodies to *Chlamydia psittaci* may be due to the low incidence (20%) of these antibodies in the control group. The incidence of the other antibodies tested varied from 33% for *Mycoplasma pneumoniae* to 97% for VCA-IgG.

Our study does not exclude the possibility that primary EBV-infections may induce cold urticaria. However, in most patients with chronic cold urticaria there seems to be no relationship with EBV. Our findings with regard to HSV, CMV, measles virus, *Mycoplasma pneumoniae* and VZV do not necessarily imply a causal relationship between cold urticaria and any of these organisms. It is well known that latent HSV, VZV and CMV may become re-activated in immunocompromised patients. However, to our knowledge, this has never been described for *Mycoplasma pneumoniae* and, apart from patients with subacute sclerosing panencephalitis (SSPE), neither for measles. Interestingly, the high titres for measles virus antibodies (⩾1:128) found in our study occurred even in patients of 55 and 79 years of age.

The raised antibody levels, often to different viral and other antigens may reflect a generally heightened antibody response rather than an immune response to specific aetiological agents. A general antibody response may be caused by a basic immuno-regulatory defect, which may also be responsible for the development of cold urticaria. Such a mechanism might also explain the findings that patients with active cold urticaria, and those with negative skin tests to cold, showed very similar serological patterns. The validity of the assumptions could be tested by measuring antibody responses to non-infectious agents.

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# Photoageing: the role of UVB, solar-simulated UVB, visible and psoralen UVA radiation

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## SUMMARY

We have studied the morphological and histological effects of chronic exposure of two strains of haired mice (albino Balb/c and pigmented C3H-) to various types of radiation. UVB (280–320 nm) radiation from unfiltered sunlamp bulbs for 20 and 30 weeks produced marked epidermal acanthosis and dyskeratosis which was reversible in mice exposed for 20 weeks followed by a 20-week rest. In the dermis the elastic fibres were altered and in the pigmented mice these changes had almost completely reversed 29 weeks after cessation of exposure. Cellulose acetate-filtered sunlamp bulbs (>289 nm) produced similar but less marked changes. Chronic exposure to UVA (320–400 nm) radiation produced minimal alterations in the dermis while the epidermis was normal. Visible (>400 nm) radiation in large doses produced no degenerative changes. Methoxsalen/UVA radiation produced epidermal acanthosis, dermal sclerosis, and alteration of elastic fibres, which was prominent in both strains at 40 weeks. These findings suggest that the UVB component of sunlight is largely responsible for photoageing of the skin. Furthermore, chronic exposure to methoxsalen/UVA therapy is likely to potentiate solar-induced photoageing.

Chronic exposure to sunlight results in gross and microscopic changes in human skin that contribute to normal ageing (Kligman, 1969; Gilchrest *et al.*, 1983). Ageing of the skin is an undesirable occurrence in our society but the best method of prevention, namely avoidance of exposure to sunlight, is equally undesirable for many people. Regular use of sunscreens might be an effective alternative for preventing photoageing, and might also permit reversal of established changes. Before such use of sunscreens is advocated it is necessary to know whether the available sunscreens have absorption spectra that include the action spectrum for photoageing. The absorption spectra of sunscreens are known but there is little information of the action spectrum for photoageing in human skin. Secondly, it is essential to know whether

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photoageing is reversible. Since it is very difficult to investigate these questions in humans, experimental animal models, principally the hairless mouse, have been used. Ultraviolet (UV) radiation from sunlamp bulbs has been shown to produce photoageing in hairless mouse skin, which is reversible (Kligman, Akin & Kligman, 1982) and which can be prevented by prior application of a sunscreen (Kligman, Akin & Kligman, 1983; Snyder & May, 1975). However, studies using sunlamp bulbs as the source of radiation can only suggest the likely effects of sunlight because the two sources have very different emission spectra. Sunlamps mainly emit radiation less than 320 nm, have a small emission in the UVA (320–400 nm) waveband and little emission at longer wavelengths. In contrast, sunlight at the surface of the earth contains little radiation less than 300 nm, and large amounts of radiation in the UVA and visible (400–800 nm) regions.

In this study we have investigated whether radiation from sunlamp bulbs produces changes of photoageing in haired mice that are similar to the changes observed in hairless mice; whether large doses of UVA and visible radiation produce photoageing, and whether any changes which are produced are reversible. In addition, we have examined the ageing effects of methoxsalen and UVA radiation (PUVA treatment) on mouse skin.

## METHODS

### *Animals*

Specific-pathogen-free female mice of the inbred haired strains C3H/HeNCR-(MTV-), a pigmented strain and BALB/cAnNCR, an albino strain, were supplied by the NIC Frederick Cancer Research Facility. The mice were 6 weeks old at the start of the experiment. They had free access to Purina Mouse Chow and chlorinated water and were housed in rooms where the ambient lighting was automatically regulated on a 12-hour light/dark cycle.

### *Exposure to radiation*

The dorsal fur was shaved with electric clippers immediately prior to the first expoure to radiation and each week thereafter. Groups of eight mice were treated as follows:

UVB radiation: A 1 h exposure three times weekly to a bank of six FS40 sunlamps (Westinghouse, Bloomfield, NJ, U.S.A.).

Solar-simulated UVB radiation: A 90 min exposure 3 times weekly to a bank of six FS40 sunlamps filtered through a 0·05 mm sheet of cellulose acetate to eliminate radiation < 289 nm.

UVA radiation: An 18 h exposure daily to a bank of six UVA fluorescent bulbs (Sylvania, Danvers, MA, U.S.A.) filtered through two 0·05 mm sheets of Mylar to eliminate radiation < 315 nm.

Visible radiation: An 18 h exposure daily to a bank of six cool white F40 CW fluorescent bulbs (Sylvania) filtered through a 0·05 mm sheet of weathered Mylar to eliminate radiation < 390 nm.

PUVA: Methoxsalen 0·4 mg (Paul B. Elder Co., Bryan, OH, U.S.A.) was given intraperitoneally 30 min before a 20 min exposure to UVA radiation 3 times weekly. After 12 weeks of treatment the exposure of BALB/c mice was reduced to 10 min because of marked scabbing.

The radiation sources were 20 cm from the animals. Animals treated with UVB, solar-simulated UVB and PUVA were housed under normal ambient conditions when not exposed to radiation. Animals treated with visible and UVA radiation were housed in the dark for the 6 h daily that they were not exposed to radiation. Control shaved and unshaved mice were housed in normal ambient conditions. Four pigmented and four albino animals, two shaved and two unshaved, were sacrificed after 20 and after 40 weeks.

### *Radiometry*

The irradiances of each radiation source and of room lighting were measured with a spectroradiometer (Model 742, Optronic Labs., Inc., Orlando, FL, U.S.A.) interfaced with a minicomputer (HP9915/S, Hewlett Packard, Fort Collins, CO, U.S.A.). At the start of the experiment the irradiances at the level of the backs of the mice were: UVB, 1 $W/m^2$ of wavelengths $<320$ nm; solar-simulated UVB, 0·7 $W/m^2$ of 289–320 nm radiation; UVA, 4·2 $W/m^2$ of 315–400 nm radiation; visible light 18 $W/m^2$ of 390–800 nm radiation. The UVA and visible bulbs were changed when the irradiances had decreased by 50%. The irradiance of ambient room lighting was 1·6 $W/m^2$ in the 400–800 nm waveband.

### *Procedure*

Two pigmented and two albino animals from each group were sacrificed after 20, 30 and 40 weeks of treatment and the dorsal skin was removed for histological examination. In addition, the treatment of two pigmented and two albino from each group was stopped after 20 weeks and these mice were kept under ambient conditions before sacrifice 20 weeks later.

### *Histology*

A strip of dorsal skin, approximately 1 × 2 cm taken at right angles to the long axis was fixed in 10% buffered formalin. Sections were cut at 7 $\mu m$ and stained as follows: haematoxylin and eosin (H & E), acid orcein and Verhoff van Gieson for elastic fibres; colloidal iron for acid mucopolysaccharides; Periodic acid-Schiff (PAS) with diastase digestion for neutral mucopolysaccharides; Fontana-Masson for melanin, and reticulin stain.

## RESULTS

### *Gross morphological appearance of the dorsal skin*

At 20 weeks, mice treated with UVB radiation showed alopecia, scaling and occasional ulcerations, while those treated with solar-simulated UVB radiation and PUVA had similar but less marked changes. The animals exposed to UVA and visible radiation appeared normal.

At 40 weeks, mice treated with UVB, solar-simulated UVB radiation, and PUVA had almost complete alopecia with areas of scarring and small areas of ulceration. Tumours developed in both strains of mice that received these treatments. The animals exposed to UVA and visible radiation had extensive alopecia but otherwise appeared normal.

### *Microscopic findings*

*Controls.* The histological features of normal mouse skin stained with haematoxylin and eosin are shown in Figure 1a and b. Shaving of the mice did not produce any alterations.

*UVB radiation.* Epidermal and dermal changes were present in both strains of mice after 20 weeks of irradiation (Fig. 1c and d, Table 1). Hyperkeratosis, parakeratosis, hypergranulosis, acanthosis, and dyskeratosis were prominent. Scattered atypical keratinocytes in the lower Malphigian layer were noted. The basement membrane (PAS stain) was 2–3 times normal thickness, and although this change was diffuse it was most marked in areas of severe epidermal and dermal change. The dermis was slightly thickened with denser collagen and more numerous

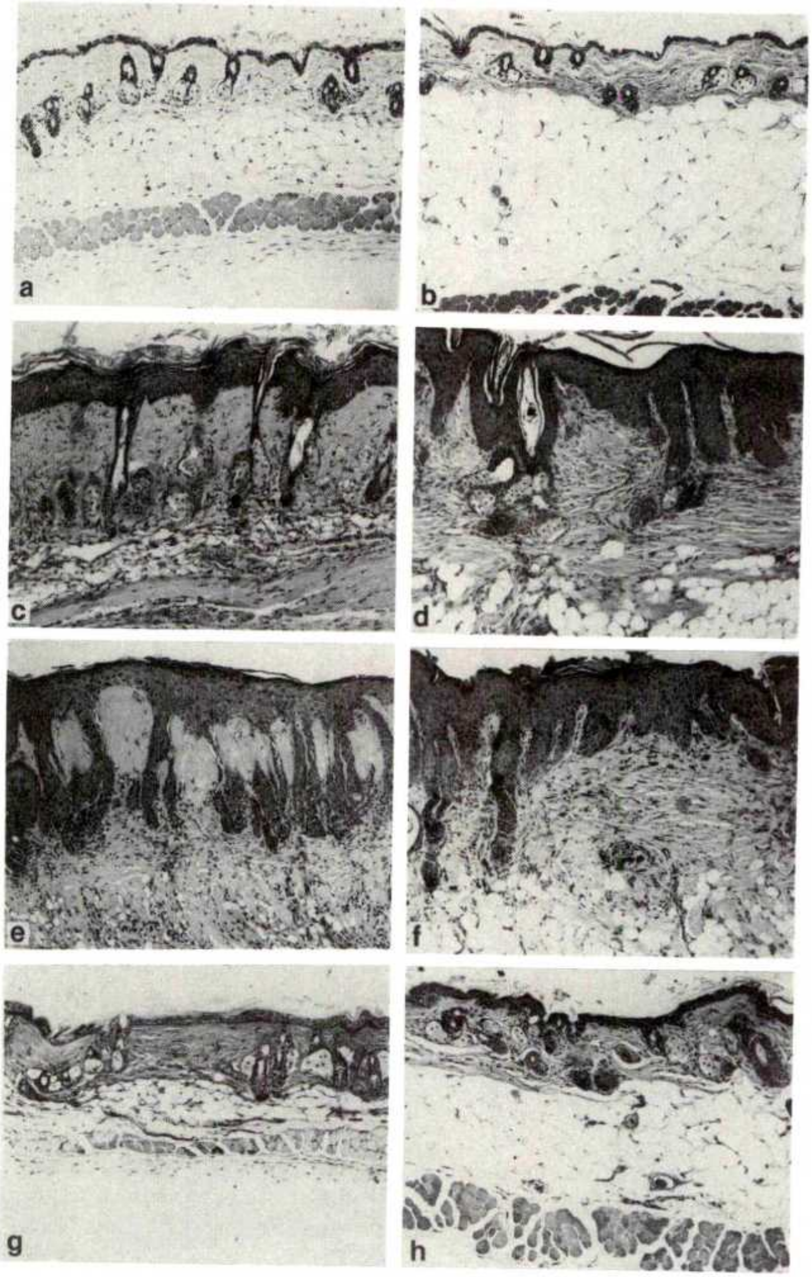

FIGURE 1. Haematoxylin and eosin-stained sections from control and UVB exposed mice. (a) control albino and (b) control pigmented mice. (c) albino and (d) pigmented mice after 20 weeks UVB irradiation. (e) albino and (f) pigmented mice after 30 weeks of UVB irradiation. (g) albino and (h) pigmented mice exposed to UVB irradiation for 20 weeks followed by 20 weeks of no exposure. (All × 135).

Table 1. Summary of histological changes graded by two observers in mice exposed to UVB irradiation

| | UVB treatment | | | | | |
|---|---|---|---|---|---|---|
| | 20 weeks | | 30 weeks | | 20 weeks' treatment 20 weeks' rest | |
| Histological change | Albino | Pigmented | Albino | Pigmented | Albino | Pigmented |
| Acanthosis | + + + | + + + | + + + | + + | – | – |
| Dyskeratosis | + + + | + + + | + + + | + + + | – | – |
| Hyperkeratosis | + + + | + + | + + + | + + + | – | – |
| Dermal sclerosis | + + + | + + + | + + + | + + + | + | + |
| Elastosis | + + + | + + + | + +/+ + + | +/+ + | – | – |
| AMPS deposition | + + | + | + + + | + + | + | – |
| BM thickening | + | + | + | + + | + + | + + |
| Pigmentation | – | + | – | + + | – | + |

– = no change; + = slight change; + + = moderate change; + + + = marked change. (Two animals per group at each time).

fibroblasts than in control animals. Acute and chronic inflammatory cells and mast cells surrounded dermal blood vessels. Occasional epidermal inclusion cysts were found in some sections but were not a constant feature. The panniculus was thin, sclerotic, and chronically inflamed; these changes were most marked in the non-pigmented Balb/c mice. The blood vessels were increased in number and in diameter.

The acid orcein stain revealed a marked focal increase of elastic tissue with thickening, fragmentation and twisting of individual fibres, and increased fibre diameter. These changes were most prominent in the mid- dermis, away from the hair follicles and at the dermal-epidermal junction. Increased acid mucopolysaccharide deposition along the dermal-epidermal junction was seen with the colloidal iron stain; the deposition was linear but extended focally into the mid-dermis. The change was seen in both strains but was more prominent in the albino mice. Follicular and inter-follicular melanization was also observed in the pigmented strain and, although the pigment was present at all levels of the epidermis, it was most marked in the basal layers. In addition, pigment was found in dermal melanophages. Reticulin fibres appeared unchanged.

After 30 weeks of irradiation the epidermal and dermal changes observed at 20 weeks were still present (Fig. 1e and f) but the dermal elastic fibre alterations were more pronounced.

In contrast to the marked epidermal changes seen after 20 weeks of irradiation, both the pigmented and non-pigmented animals irradiated for 20 weeks then rested for 20 weeks showed only slight epidermal acanthosis (Fig. 1g and h). Dermal changes were still present but were less marked as compared with the 20 and 30 week exposure groups. In the centre of the strip the dermal collagen was denser, with both an increase in fibroblasts and more chronic inflammatory cells compared to the adjacent regions. The panniculus was thinned and focally absent in areas where the sclerotic dermis adhered directly to the muscle layer. Elastic fibres were more numerous, thicker, coarser and focally clumped in the mid dermis. The basement membrane thickening and deposition in the upper dermis persisted, and were similar in degree to those in the mice treated for 20 weeks.

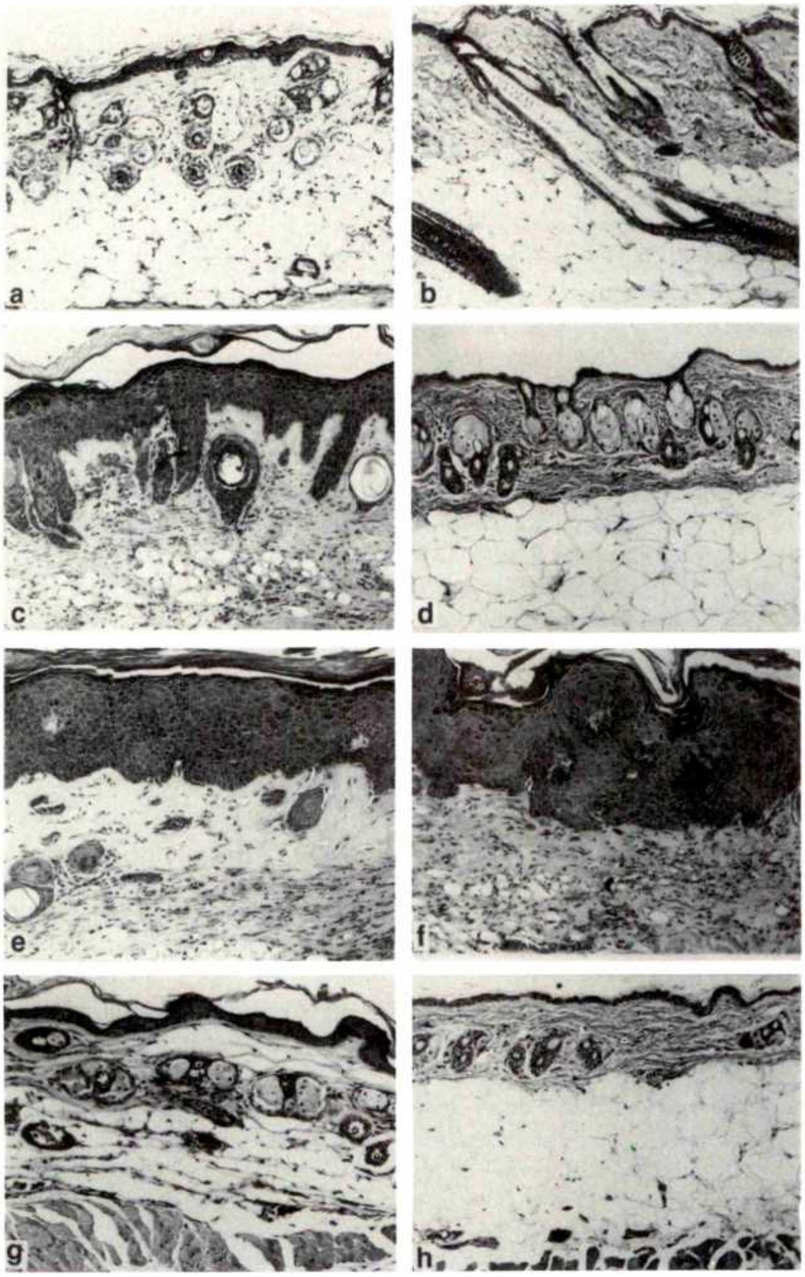

FIGURE 2. Haematoxylin and eosin-stained sections from mice treated with 8-methoxypsoralen and UVA irradiation. (a) albino and (b) pigmented mice treated for 20 weeks. (c) albino and (d) pigmented mice treated for 30 weeks. (e) albino and (f) pigmented mice treated for 40 weeks. (g) albino and (h) pigmented mice treated for 20 weeks followed by 20 weeks with no treatment. (All × 165).

*Solar-simulated UVB radiation.* After 20 weeks of exposure to UVB radiation filtered through cellulose acetate (eliminating wave lengths below 289 nm) both strains demonstrated hyperkeratosis, parakeratosis, acanthosis, and dyskeratosis. Thickening of the dermis, increased density of the collagen, and chronic inflammation were seen. Epidermal inclusion cysts, and focal replacement of the panniculus by scar tissue were found in non-pigmented mice. Elastic fibres were increased in number and width but no clumping was evident. Acid mucopolysaccharides were deposited in the upper and lower dermis.

After exposure for 30 weeks the changes listed above were more pronounced, especially the epidermal acanthosis and dyskeratosis. Elastic fibres were now aggregated into clumps and more acid mucopolysacchrides were deposited in the dermis. The epidermal and dermal changes were reversed partially in the skin of mice rested for 20 weeks after 20 weeks of irradiation. Deposition of acid mucopolysaccharides persisted only in areas of inflammation. Thickening of the basement membrane did not regress.

*UVA radiation.* The only changes noted in this group were mild chronic inflammation in the dermis and increased acid mucopolysaccharides in the upper dermis near the dermal-epidermal junction and in the lower dermis. No alterations of the basement membrane or epidermis were seen. No detectable difference was found between the 20 and 40 week groups, and the changes were still present after the 20 week rest period.

*Visible radiation.* The mice exposed to visible light did not differ from the control animals.

*PUVA treatment.* The non-pigmented strain treated with PUVA for 20 weeks showed only a mild deposition of acid mucopolysaccharides in the dermis, and prominent thickening of the basement membrane. These changes were not seen in the pigmented strain (Fig. 2a and b, Table 2).

TABLE 2. Summary of histological changes graded by two observers in mice exposed to PUVA

| | PUVA treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 20 weeks | | 30 weeks | | 40 weeks | | 20 weeks' treatment 20 weeks' rest | |
| Histological change | Albino | Pigmented | Albino | Pigmented | Albino | Pigmented | Albino | Pigmented |
| Acanthosis | – | + | + + + | – | + + + | + + | + | – |
| Dyskeratosis | – | – | + + + | – | + + + | + + | – | – |
| Hyperkeratosis | – | + | + + + | – | + + + | + + | + | – |
| Dermal sclerosis | – | – | + + | – | + + | + + | – | – |
| Elastosis | – | – | + + + | – | + + + | + | – | – |
| AMPS deposition | + | – | + + | + | + + | + | – | – |
| BM thickening | + + | – | + + | – | + + | + + | – | – |
| Pigmentation | – | – | – | – | – | – | – | – |

– = no change; + = slight change; + + = moderate change; + + + = marked change. (Two animals per group at each time).

At 30 weeks the non-pigmented strain showed marked diffuse hyperkeratosis, acanthosis, and numerous dyskeratotic cells at all layers of the epidermis and in hair follicles. The pannicular septae were fibrotic and in areas the dermis was bound down to the perimuscular soft tissue (Fig. 2c). Increased elastic fibres were present when compared with the 20 week group. Routine H & E stained sections from the pigmented mice did not differ from the controls (Fig. 2d). Both strains showed deposition of acid mucopolysaccharides in the upper and lower dermis, and an increased number of mast cells. Only the albino strain had significant thickening of the basement membrane.

Further progression of these changes was observed in the albino strain after 40 weeks of treatment and in addition the pigmented strain showed similar degrees of epidermal and dermal change (Fig. 2e and f). Dyskeratotic cells were present throughout the epidermis and in the non-pigmented strain focal epidermal necrosis and separation at the dermal-epidermal junction were seen. The elastotic change was more pronounced in the albino mice, with a change in orientation of the fibres from being parallel to being perpendicular to the surface. Both species had increased vascularity in the region of the sclerotic panniculus.

The changes in the 20 weeks of exposure/20 weeks of rest groups differed between the albino and the pigmented strains (Fig. 2g and h). Mild hyperkeratosis and acanthosis were present in the albino strain. The pigmented mice did not differ substantially from the controls or the 20 and 30 week PUVA treatment groups. Neither strain showed thickening of the basement membrane or abnormal deposition of acid mucopolysaccharides. The Fontana Mason stain did not reveal increased melanin pigment in any of the pigmented C3H-mice treated with PUVA.

## DISCUSSION

In this study both albino and pigmented haired mice irradiated with UVB or solar-simulated UVB developed alterations of the epidermis and dermis. These included parakeratosis, hyperkeratosis, acanthosis, thickening of the basement membrane, dermal sclerosis, an increase in elastic fibres, deposition of acid mucopolysaccharides, and a mild chronic inflammatory cell infiltrate. These histological changes were present in mice irradiated for 20 weeks and were more marked in mice treated for 30 weeks. However, the changes induced by 20 weeks of exposure were partially reversed after 20 weeks of rest. The residual changes noted were dermal sclerosis, thickening of the basement membrane, and hyper-pigmentation. The changes observed in the dermis of these haired mice were similar to those described in hairless and in haired mice by other workers (Kligman *et al.*, 1983; Berger, Tsambos & Mahrle, 1980; Sams, Smith & Burk, 1964). Following comparable chronic exposure to UVB radiation, Kligman *et al.* (1983) observed thickening of the dermis with increased elastic fibres, reticulin fibres, accumulation of mucopolysaccharides, and numerous large epithelial-lined cysts that filled the dermis. Increased reticulin fibres and significant numbers of epithelial cysts were not observed in the haired mice in our study.

Albino and pigmented haired mice exposed to UVA radiation exhibited only mild chronic inflammation and deposition of mucopolysaccharides in the dermis. This finding contrasts with the report of Berger *et al.* (1980) who exposed hairless mice to broadband UVA radiation for more than 30 weeks. They observed marked changes in the dermis and epidermis. However, although the total doses of UVA radiation were similar in both Berger's and our studies, the radiation sources were quite different. Berger *et al*, (1980) did not remove wavelengths shorter than 320 nm and the effects they observed were probably due to the UVB radiation emitted by the lamps.

Treatment of haired mice with methoxsalen and UVA radiation produced histological alterations in the skin that were similar to those seen in UVB-irradiated mice. The changes were largely in agreement with those previously reported to occur in hairless mice after chronic exposure to PUVA (Langner, Wolska & Marzulli, 1977) although alteration of elastic tissue was not observed in that study. Exposure of mice to large doses of visible radiation for up to 40 weeks resulted in extensive alopecia but there were no abnormal findings on histological examination of the skin.

Photoageing is partially reversible in mice. Both pigmented and non-pigmented mice examined after 20 weeks of rest following 20 weeks of exposure to UVB radiation showed the return of epidermal thickness to normal, less dermal elastotic change and less perivascular inflammation. It is interesting that the mild changes in UVA-irradiated mice were not reversible.

The results of this study allow us to draw some tentative conclusions about the photobiology of ageing in mice. First, the radiation from sunlamp bulbs is most effective in producing these changes, and elimination of wavelengths shorter than 290 nm does not greatly diminish the effect. Second, chronic exposure to UVA radiation in the absence of a photosensitizer does not produce marked histological changes but in the presence of a photosensitizer such as methoxsalen, the changes can be comparable to those produced by UVB radiation. Third, chronic treatment with visible radiation does not produce histological changes in the skin. Finally, photoageing is partially reversible. Extrapolation of these results to humans would suggest that the 290–320 nm waveband of sunlight is likely to be mainly responsible for photoageing. Therefore, commercially available sunscreens should be effective in preventing this degenerative process since this is the waveband absorbed by most of these preparations. Furthermore, photoageing of human skin might be partially reversed by avoidance of sunlight or consistent use of an effective sunscreen.

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# Light and scanning electron microscopic studies on wrinkles in aged persons' skin

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## SUMMARY

Wrinkles in six aged persons (67–82 years of age) have been investigated by light microscopy (LM) and scanning electron microscopy (SEM). There are two types of wrinkles. One is a deep wrinkle which develops on the sun-exposed skin and does not disappear on stretching (permanent wrinkle). The LM and SEM showed less elastotic change in the upper dermis in the area of wrinkle than in that of the surroundings. The other type is a shallow wrinkle which develops on sun-protected skin and disappears on stretching (temporary wrinkle). The LM and SEM showed the decrease or loss of the elastic fibres in the papillary dermis as seen in ageing skin.

Wrinkles are the most commonplace of all the signs of ageing. However, there have been few studies on wrinkles except for those which form in some congenital or acquired skin disorders (Verhagen & Woerdeman, 1975; Shelley & Wood, 1977; Brenner *et al.*, 1978). In general, elastic fibres have been suggested to play an important role in the formation of wrinkles.

In the present study we investigated the elastic fibres in wrinkles using light and scanning electron microscopy.

## METHODS

### *Subjects*

Wrinkles of six aged persons (67–82 years of age, four men and two women) were investigated. They had many deep wrinkles occurring symmetrically on the face and neck (Fig. 1), most of which did not disappear on stretching transversely to the direction of the wrinkle line (Fig. 2). They also had fine shallow wrinkles on the abdomen and buttocks (Fig. 3), which easily disappeared on stretching (Fig. 4).

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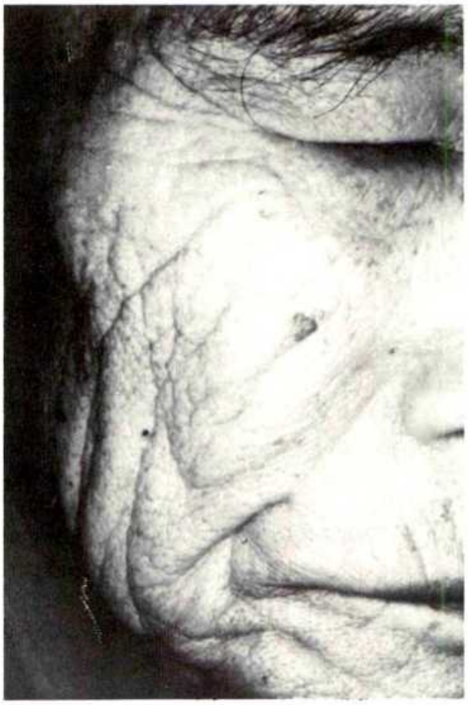

FIGURE 1. Deep wrinkles on the face of an 80-year-old man.

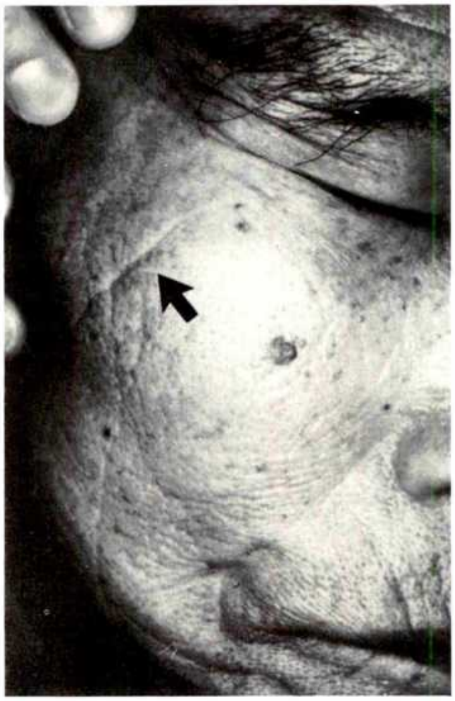

FIGURE 2. The deep wrinkles on this man's face (arrow) do not disappear on stretching.

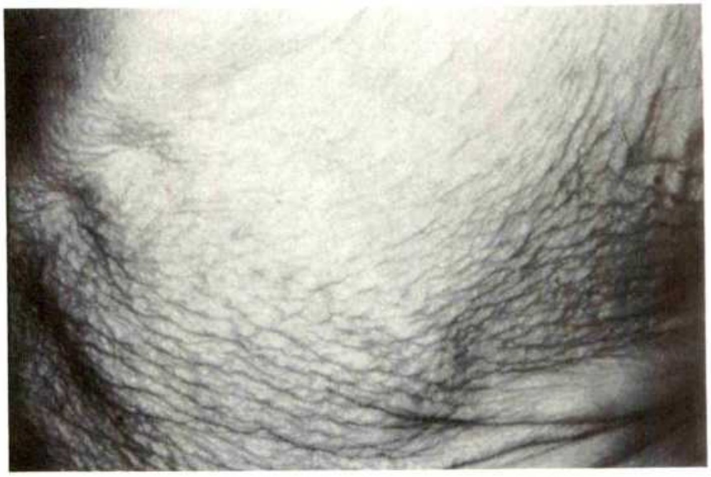

FIGURE 3. Shallow wrinkles on the abdomen of an 82-year-old man

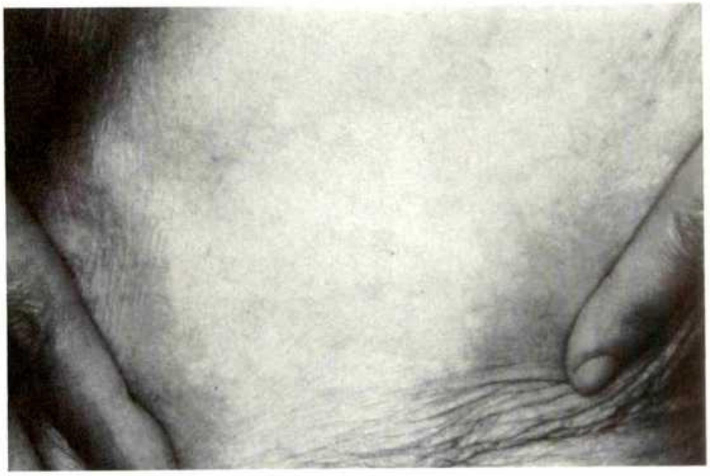

FIGURE 4. Shallow wrinkles on this man's abdomen disappear on stretching.

*Biopsies*

Skin biopsy specimens which included the deep or shallow wrinkles were taken from each person. All the specimens were processed for light microscopy (LM) and scanning electron microscopy (SEM). For LM, the specimens were fixed in 10% formalin and embedded in paraffin. Cross sections of the wrinkles were cut and stained with haematoxylin-eosin, Weigert, van Gieson, and elastic-van Gieson techniques.

For SEM, a method we have developed was employed (Tsuji, Lavker & Kligman, 1979). The specimens were cut approximately 1 mm thick to expose cross sections of the wrinkles. These sections were placed in distilled water and autoclaved for 8 h at 121 °C and a pressure of 18 lb/in$^2$. The autoclaved specimens were fixed at 4 °C in 2% glutaraldehyde in 0·1 mol/l phosphate buffer pH 7·4 for 1 h, then rinsed in the buffer for 1 h. After dehydration in a graded series of ethanol baths (70–100%), the samples were placed in 100% xylol for 2 h, and then air dried. The dried samples were mounted on aluminium stubs using cellophane tape. They were coated with a thin layer of gold palladium in a sputter coater and viewed in a Hitachi S-800 scanning electron microscope operated at 5–15 kV.

## RESULTS

### *Light microscopic findings*

In the sections from the face and neck, the epidermis was thin and flattened. The upper portion of the dermis showed the marked degeneration known as solar elastosis, which stained basophilic with haematoxylin-eosin, and pale pink with van Gieson. With Weigert or elastic-van Gieson stains, many thickened tightly packed fibres were seen. In the area of the wrinkle, the epidermis was often depressed and the upper portion of the dermis showed much less degeneration than the surroundings; the thickness of the elastotic degeneration was decreased to between two thirds and a half of that of the sourroundings, and intact collagen was seen widely above or within the elastotic lesion (Figs. 5 and 6).

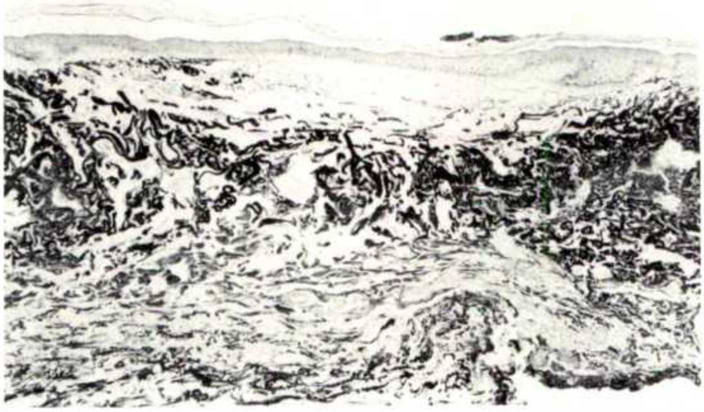

FIGURE 5. Light microscopy of a deep wrinkle on the neck of an 80-year-old man. Note broad intact dermis just below the epidermis and much less elastotic degeneration in the area of the wrinkle (centre) than in the surroundings. (Weigert stain, original × 100).

In the sections from the abdomen and buttock, the elastic fibres in the papillary dermis (terminal fibres and arcades) were decreased or had disappeared (Fig. 7). Elastotic changes were not seen, nor were any differences in elastic fibres between the wrinkle and the surroundings observed throughout the dermis. The elastic fibres in the reticular dermis were decreased in some subjects, while they were increased in others. No focal loss of the elastic tissue was seen in the reticular dermis of any of the subjects.

### *Scanning electron microscopic findings*

In the area of the wrinkles of the face and neck, relatively normal-looking but irregularly running elastic fibres separated by large spaces were seen in the upper portion of the dermis (Fig. 8). Moving away from the area of the wrinkle, thickened and tortuous elastic fibres were increased, and spread to the middle dermis, forming a thick layer of compact fibres with small spaces between them (Fig. 8).

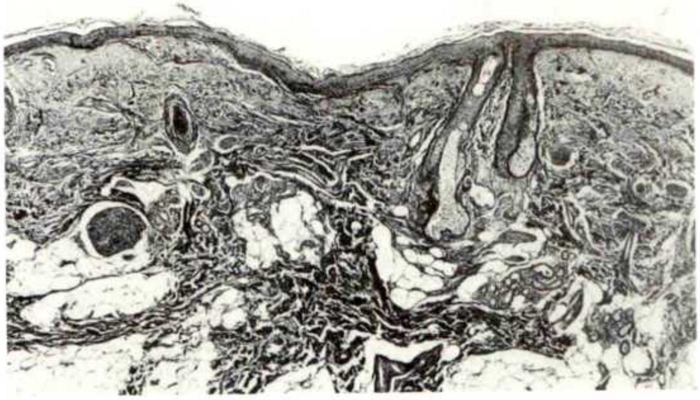

FIGURE 6. Light microscopy of a deep wrinkle on the face of an 80-year-old woman. Note depressed epidermis and much less elastotic degeneration intermingled with intact collagen in the area of the wrinkle (centre) than in the surroundings. (van Gieson stain, original × 100).

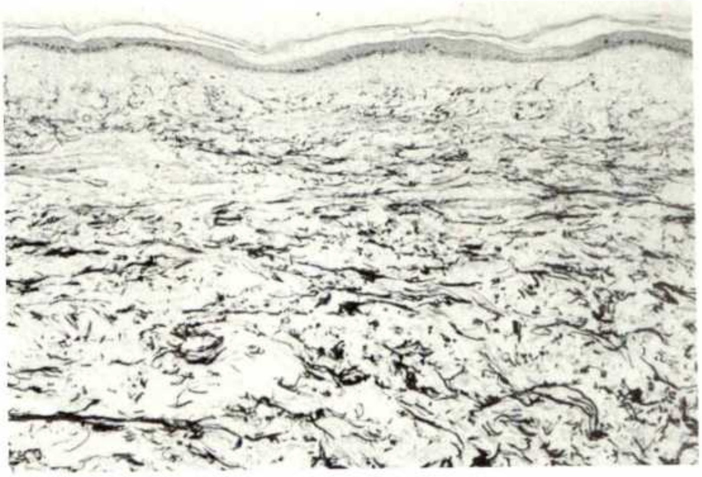

FIGURE 7. Light microscopy of a shallow wrinkle on the abdomen of an 82-year-old man. Note fewer elastic fibres in the papillary dermis and no elastotic changes throughout the dermis. (Weigert stain, original × 100).

In the sections from the abdomen and buttock, the elastic fibres in the upper portion of the dermis were very thin and curly, running in different directions (Fig. 9). The elastic fibres in the middle and deep dermis were similar to those in aged skin reported previously (Tsuji & Hamada, 1981). No difference was seen in the elastic fibres between the area of the wrinkle and the surroundings, throughout the dermis.

## DISCUSSION

The present study has shown, on the basis of clinical and histological findings, that there are two types of wrinkles in aged persons' skin. One is a deep wrinkle which develops on sun-exposed

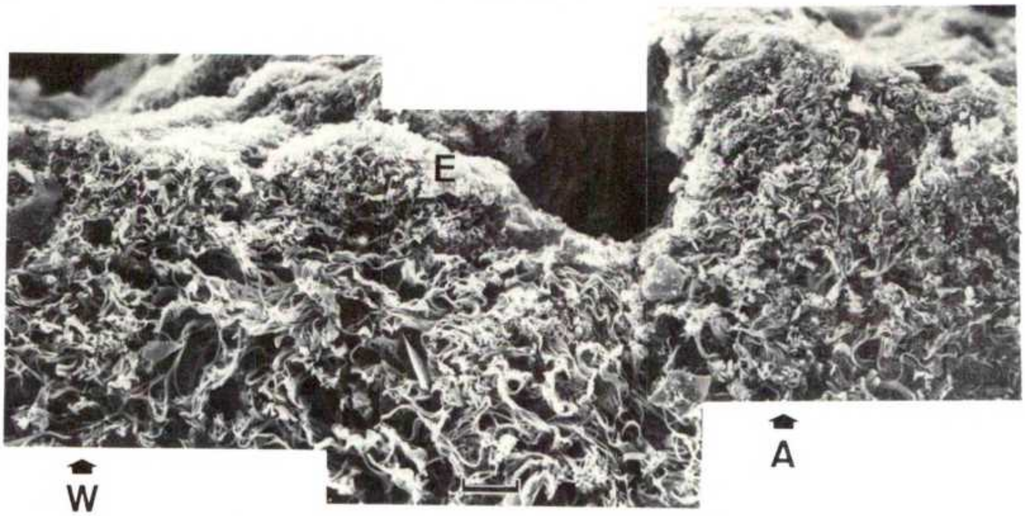

FIGURE 8. Scanning electron microscopy of a deep wrinkle on the face of an 80-year-old woman. Note relatively normal-looking but irregularly running elastic fibres separated by large spaces in the area of the wrinkle (W), and an increased amount of thickened and tortuous elastic fibres leaving little space between them, around the wrinkle (A). (E: epidermis, Bar: 50 μm).

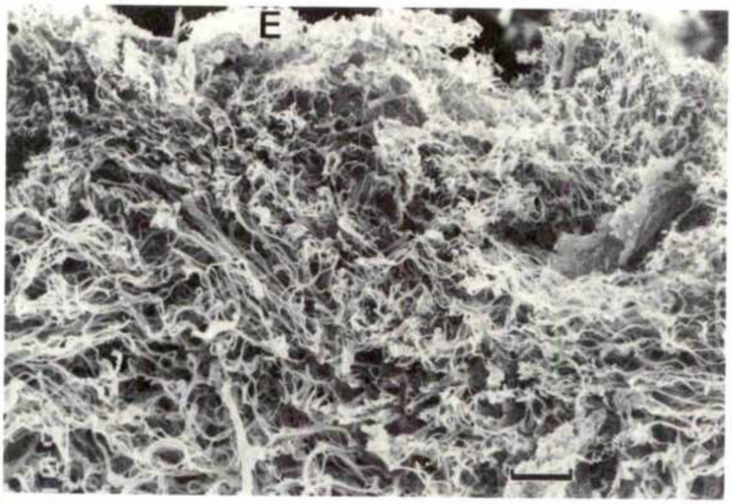

FIGURE 9. Scanning electron microscopy of a shallow wrinkle on the abdomen of an 82-year-old man. Note very thin and curly, irregularly running elastic fibres with no difference between those in the area of the wrinkle and in the surroundings, throughout the dermis. (E: epidermis, Bar: 50 μm).

skin, such as the face and neck, and does not disappear on stretching. The LM and SEM have demonstrated that in the upper portion of the dermis the elastic fibres are thick and tortuous, having undergone elastotic changes which are less in the area of the wrinkle than the surroundings. The other is a fine, shallow wrinkle which develops on the sun-protected skin, such as abdomen and buttock, and which disappears on stretching. The LM has demonstrated that the elastic fibres in the papillary dermis are completely or almost completely absent, and the

SEM has shown that there are thin and curly elastic fibres in the upper portion of the dermis. There were no differences between the area of the wrinkles and the surroundings. We propose to call the deep wrinkles 'permanent wrinkles' and the shallow ones 'temporary wrinkles'.

We suggest the following mechanism of formation of the two types of wrinkles. The facial and neck skin responds to every movement of the underlying muscles in smiling, frowning and physical movement. The movement produces temporary but repeated wrinkling of the same portion of the face or neck. This facility of response is achieved by the presence of a remarkably complex and dense intradermal elastic tissue mesh. With years of sunlight exposure, there comes the degeneration of elastic tissue known as solar elastosis, resulting in thickened skin. It seems reasonable to assume that the wrinkles on the face and neck are subjected to less damage than the surroundings because of less sunlight exposure. This results in permanent wrinkles like a valley between the areas of thickened skin, and they no longer disappear on stretching. The LM and SEM findings support this as there were marked elastotic changes surrounding the area of the wrinkles which themselves showed less elastotic change. Thus, this type of wrinkle is due to a biophysical rather than a histological change, but is closely associated with the above morphological abnormalities of the dermal elastic fibres.

On the other hand, the sun-protected skin, particularly the abdomen and buttock skin, becomes thin with a decrease in subcutaneous fat tissue with age, and wrinkles easily. Most of the wrinkles are fine and shallow. The movement of the underlying muscle does not cause the wrinkles, but simply makes them evident. These are not permanent wrinkles since they easily disappear on stretching. The LM findings in these wrinkles are identical to the changes seen in ageing skin (Montagna, 1973; Montagna & Carlisle, 1979). In addition, the SEM findings suggest that the elastic fibres in the papillary dermis are not absent, but have become too thin to be clearly recognized by LM.

Previous investigators have reported wrinkles due to idiopathic or non-inflammatory elastolysis, the histology of which shows loss of mid-dermal elastic fibres (Shelley & Wood, 1977; Brenner *et al.*, 1978), and wrinkles due to post-inflammatory elastolysis, the histology of which shows almost complete loss of the upper and mid-dermal elastic fibres with the remaining elastic fibres fragmented or swollen (Verhagen & Woerdeman, 1975). These wrinkles are different from either of the two types of wrinkles in aged persons' skin described here.

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# Alopecia areata: alterations in the hair growth cycle and correlation with the follicular pathology

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## SUMMARY

A histopathological study was performed in 17 patients with alopecia areata to elucidate the changes in hair cycle dynamics. The findings confirm the view that the initial event in alopecia areata is the premature entry of anagen follicles into telogen, although some follicles survive for a time in a dystrophic anagen state. However, after re-entry into anagen takes place, growth appears to be halted in anagen III rather than anagen IV, as has previously been suggested. Follicles then return prematurely to telogen and these truncated cycles are repeated until the disease activity subsides. A new pathogenic hypothesis is presented which relates alterations in hair cycle dynamics to pathological changes within the anagen follicle and also provides an explanation for the formation of exclamation mark hairs and the non-destructive nature of the disease.

Alopecia areata is known to cause an alteration in the normal dynamics of the hair cycle. Eckert, Church and Ebling (1968) examined the early events in alopecia areata by plucking hairs from concentric circles around bald patches. They concluded that the disease progresses as a wave of follicles prematurely entering telogen and this view is generally accepted. However, the subsequent events have been the subject of conflicting reports. Van Scott (1958) studied biopsies from established bald patches, some of many years duration, and found that up to 75% of follicles were in anagen, suggesting that re-entry into anagen takes place. Anagen follicles were diminutive and their development appeared to be restrained at a stage equivalent to anagen IV of the mouse hair cycle (Chase, Rauch & Smith, 1951). In contrast, Swanson *et al.* (1981) reported that in longstanding disease there is total telogen transformation suggesting that alopecia areata causes telogen arrest.

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In a previous publication (Messenger & Bleehen, 1984) we reported that lesional anagen follicles may show injury confined to keratinocytes in the presumptive cortex and we suggested that this represents a primary pathogenetic event. In the present paper we report the findings of a histopathological study designed to clarify the changes in normal hair cycle dynamics that occur during the natural history of alopecia areata. The relation of these findings to the pathology within the hair follicle is discussed. The proposed hypothesis is used to explain the formation of exclamation mark hairs and the non-destructive nature of the disease.

## METHODS

Scalp biopsies were taken, with informed consent, from 17 patients with alopecia areata. None had received treatment for at least 3 months before the biopsy. Seven biopsies were from the edges of developing bald patches, eight from alopecia totalis or from the centre of established bald patches and two from patients showing regrowth of fine fuzzy hair.

The biopsy specimens were fixed in phosphate-buffered 10% neutral formalin and processed routinely to paraffin wax for light microscopy. Seven biopsies were divided into two pieces; one piece was sectioned vertically with respect to the skin surface and the other horizontally. The remaining 10 specimens were sectioned vertically. Serial 100 μm sections were taken through all the biopsies and stained with haematoxylin and eosin.

## RESULTS

Patient details are given in Table 1.

TABLE 1. Details of 17 patients with alopecia areata included in the study

| Patient | Age (years) | Sex | Disease duration | Site of biopsy | % of anagen follicles |
|---|---|---|---|---|---|
| 1 | 36 | M | 2 months | Edge of lesion | 44 |
| 2 | 36 | M | 1 month | Edge of lesion | 50 |
| 3 | 37 | F | 4 months | Edge of lesion | 50 |
| 4 | 44 | M | 1 year | Edge of lesion | 43 |
| 5 | 32 | M | 1 year | Edge of lesion | 39 |
| 6 | 54 | M | 2 months | Edge of lesion | 10 |
| 7 | 14 | F | 5 years | Edge of lesion | 0 |
| 8 | 20 | M | 2 years | Centre of lesion | 16 |
| 9 | 65 | F | 7 months | Alopecia totalis | 33 |
| 10 | 28 | M | 7 years | Alopecia totalis | 62 |
| 11 | 30 | F | 17 years | Alopecia totalis | 13 |
| 12 | 42 | M | 39 years | Alopecia totalis | 36 |
| 13 | 45 | F | 34 years | Alopecia totalis | 36 |
| 14 | 14 | F | 5 years | Alopecia totalis | 52 |
| 15 | 44 | M | 1 year | Alopecia totalis | 41 |
| 16 | 20 | M | 11 years | Early regrowth | 29 |
| 17 | 29 | F | 1 year | Early regrowth | 68 |

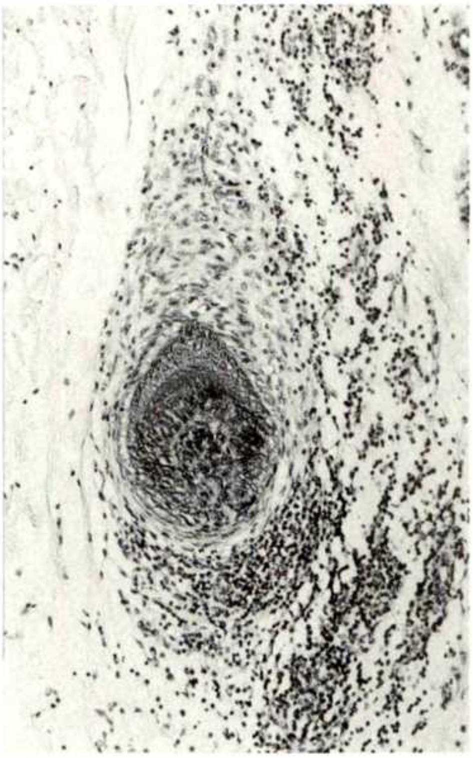

FIGURE 1. Alopecia areata: edge of developing bald patch. Anagen hair bulb in lower dermis with dense peribulbar lymphocytic infiltrate. (Original × 100).

*Biopsies from the edge of expanding bald patches*

In biopsies taken from the edges of expanding bald patches the majority of follicles were in late catagen and telogen. The proportion of follicles in catagen/telogen was at least 50% and in one biopsy was 100%. The anagen follicles present were either of normal size with the bulb situated in the lower dermis or upper sub-cutis, or smaller than normal with the bulb situated at the mid-dermal level. The larger anagen follicles all showed a peribulbar lymphocytic infiltrate which was usually intense (Fig. 1). The infiltrate sometimes contained small numbers of eosinophils and plasma cells. A prominent feature of smaller anagen follicles was the disproportionate reduction in the size of the epithelial matrix in relation to that of the dermal papilla (Fig. 2). Below the bulb of small anagen follicles a fibrous tract extended into the sub-cutis and this usually contained prominent capillaries and large clumps of melanin. These follicles also showed a peri-bulbar infiltrate but this was variable in intensity. Catagen and telogen follicles only occasionally contained club hairs but their morphology was otherwise normal. A lymphoid infiltrate was often associated with catagen follicles but this was situated predominantly in the lower dermis and sub-cutis at the site the hair bulb occupied when the follicle was in anagen (Fig. 3). Some of the infiltrate tracked up alongside the epithelial column of catagen follicles but little or no infiltrate was seen around telogen follicles.

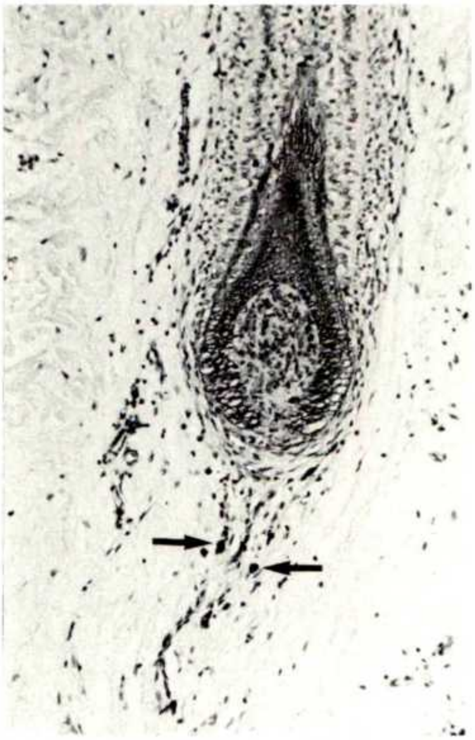

FIGURE 2. Alopecia areata: edge of developing bald patch. Anagen hair bulb in mid-dermis. Dermal papilla volume is increased relative to the epithelial matrix. A fibrous tract containing clumps of melanin (arrows) extends below the follicle. There is a light peribulbar lymphocytic infiltrate. (Original × 100).

### *Alopecia totalis and established bald patches*

Transverse sections of biopsies taken from alopecia totalis and from bald patches revealed that pilar units were present in normal numbers even in longstanding disease (Fig. 4). Both anagen and telogen follicles were present in all the biopsies, the proportion in anagen varying between 13% and 62% (Table 1). The secondary epithelial germ of telogen follicles was generally smaller than that of normal telogen follicles and this was more pronounced in longstanding disease. Anagen follicles were also smaller than normal fully-developed anagen follicles with their bulbs situated at or above mid-dermal level. A peribulbar lymphocytic infiltrate was seen around most anagen follicles but this was less dense than that around anagen follicles in early lesions and was sometimes very sparse. An infiltrate was not seen around telogen germs. Apart from an occasional peripherally-located apoptotic cell, the matrix region in the lower bulb of anagen follicles appeared normal. Mitotic figures could usually be found, although examination of several serial sections was often necessary. Some anagen follicles showed vacuolation of epithelial cells around the upper pole of the dermal papilla (Fig. 5). Anagen follicles at mid-dermal level all showed a keratinizing inner root sheath and a normal outer root sheath. Transverse sections revealed that the cross-sectional diameter of the inner root sheath progressively diminished as it ascended the follicle eventually coming to a point over the centre of the follicle overlying the cortex. Thus, in three dimensions the inner root sheath would be a

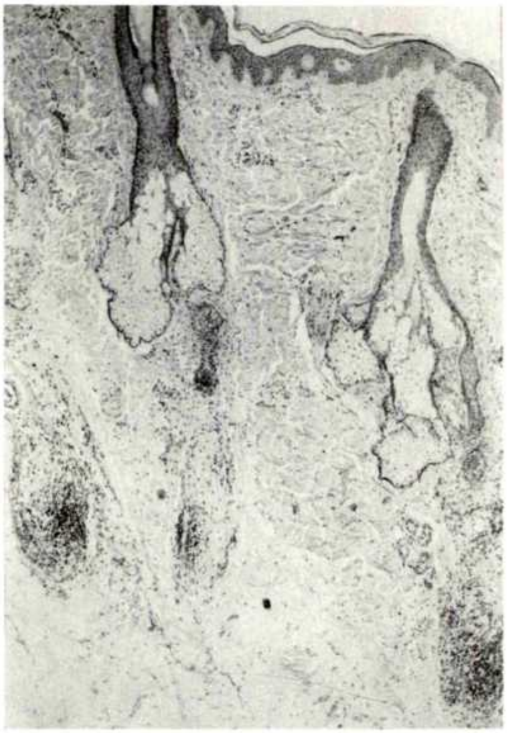

FIGURE 3. Alopecia areata: edge of developing bald patch. Two follicles in late catagen are shown. There is a lymphocytic infiltrate in the lower dermis at the site the hair bulbs occupied when in anagen. (Original × 40).

cone-shaped structure (Fig. 6). Below the inner root sheath, early differentiation of cortical cells was indicated by these cells showing elongation with their long axes aligned with the long axis of the follicle (Fig. 5) but in no case was there evidence of cortical keratinization. Medulla formation was not seen. Catagen follicles were also found although their numbers were low.

### *Biopsies from areas of hair regrowth*

In biopsies taken from areas of early regrowth, both anagen and telogen follicles were larger than those in established lesions. Anagen development had progressed to the formation of a fine non-medullated hair shaft which penetrated the inner root sheath. In one biopsy, individual follicles contained several hair shafts (Fig. 7).

In summary, the major findings illustrating changes in hair cycle kinetics in alopecia areata were as follows:

(1) Biopsies taken early in the course of the disease showed that the majority of follicles were in telogen or late catagen. Some of the anagen hair bulbs present were situated at a higher level in the dermis than normal.

(2) In established lesions there was no reduction in overall follicle numbers. Anagen follicles were identified in all the biopsies though the anagen/telogen ratio varied considerably.

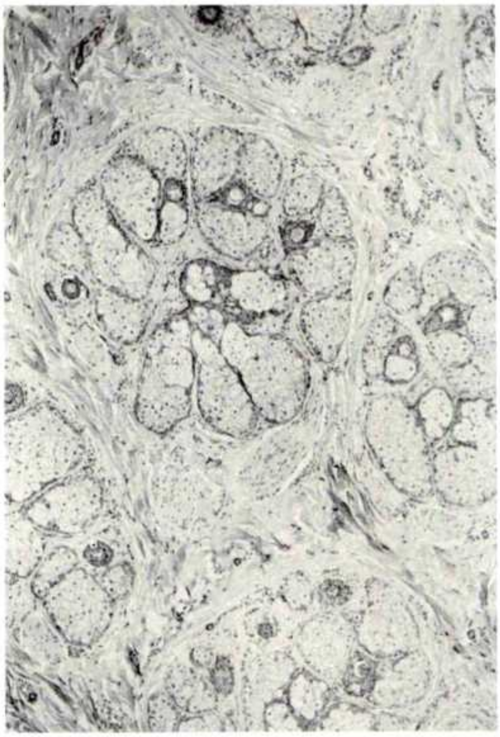

FIGURE 4. Alopecia totalis: transverse section. Pilar units are present in normal numbers. Sebaceous glands are normal. (Original × 40).

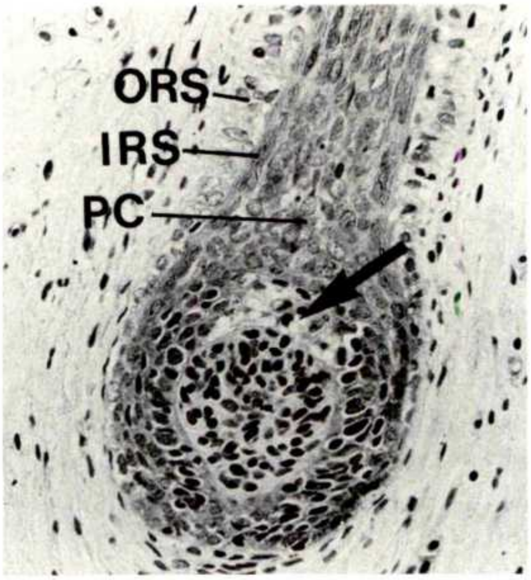

FIGURE 5. Alopecia totalis. Anagen hair bulb showing vacuolation of epithelial cells (arrow) around upper pole of the dermal papilla. Inner root sheath (IRS) and outer root sheath (ORS) are present and there is early formation of the presumptive cortex (PC). (Original × 250).

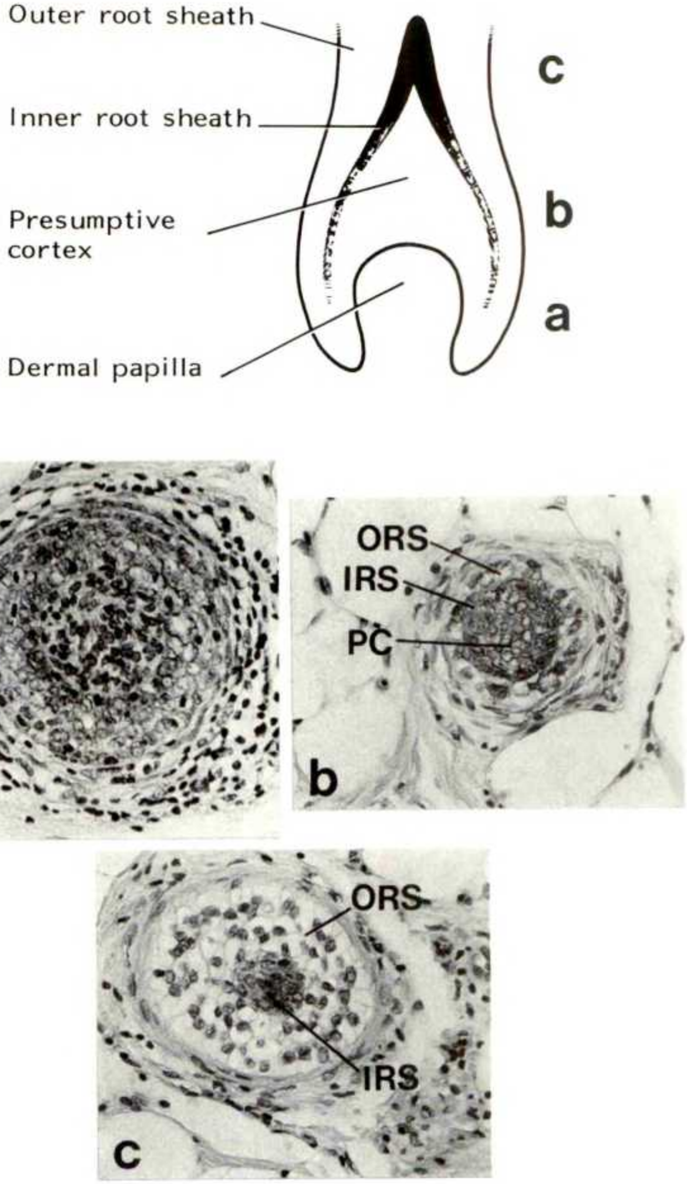

FIGURE 6. Alopecia totalis: transverse sections. The diagram illustrates a follicle in anagen III. The level of sections a, b and c in the accompanying photomicrographs is indicated. (a) Anagen follicle sectioned through matrix region. The dermal papilla can be seen in the centre. There is a peribulbar lymphocytic infiltrate. (Original × 250). (b) Anagen follicle. There is a single layer of outer root sheath cells (ORS) and a keratinizing inner root sheath (IRS). Cortical cells (PC) are not keratinized. (Original × 250). (c) Anagen follicle. The outer root sheath is multi-layered (ORS). The inner root sheath (IRS) is continuous over the centre of the follicle. (Original × 250).

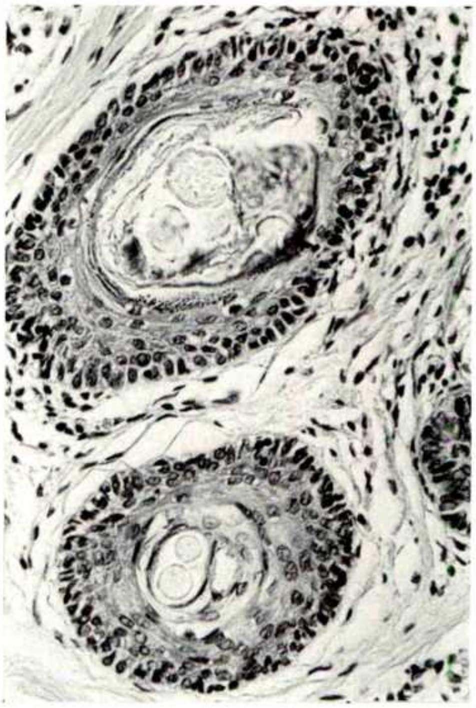

FIGURE 7. Alopecia areata: early regrowth. Transverse section through two follicles at infundibular level. Each contains two hair shafts. (Original × 250).

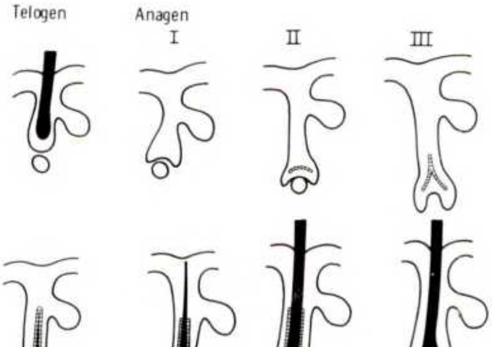

FIGURE 8. Diagram illustrating stages in anagen development. Anagen I: there is onset of mitotic activity in the matrix which begins to invest the dermal papilla. Anagen II: the inner root sheath (hatched) appears as a keratinized plate overlying the matrix. Anagen III: the inner root sheath is now a conical structure beneath which the cortex is begining to develop. Anagen IV: keratinization of the cortex has commenced. Anagen V: the hair shaft penetrates the inner root sheath at the level of the sebaceous duct. Anagen VI: the fully developed anagen follicle.

Anagen follicles were small and showed a conical root sheath with evidence of early cortical differentiation but no signs of cortical keratinization.

(3) During early regrowth some follicles contained multiple fine hair shafts.

The changes described involved the lower, transient portion of the hair follicle. In four biopsies a lymphocytic infiltrate was also seen around the upper, permanent, part of the hair follicles, irrespective of whether the follicle was in anagen or telogen. This was sometimes associated with spongiosis of the infundibular epithelium. Atrophy of sebaceous glands was seen in only one biopsy from longstanding alopecia totalis.

## DISCUSSION

The histological findings in biopsies taken from the edges of expanding bald patches were consistent with the conclusions of Eckert, Church and Ebling (1968) that alopecia areata progresses as a wave of follicles entering telogen, although some follicles may persist for a time in a dystrophic anagen state. They suggested that the anagen follicle could respond in one of three ways to the initial insult depending on its severity. If the initial insult was sufficiently severe, keratinization of the hair shaft would be impaired, causing a focal weakness in the hair which would break at that point when it reached the skin surface. At the same time the follicle was precipitated into catagen. A less severe insult resulted simply in premature entry into telogen whereas the least severely affected follicles were able to continue in anagen but produced a dystrophic hair. The entry of anagen follicles into catagen appears to be preceded or accompanied by the accumulation of a peribulbar lymphocytic infiltrate. The fibrous tract trailing below small anagen follicles in early lesions suggests that they have moved up from their original position. These follicles may be the source of dystrophic hairs. The changes in the relative proportions of the dermal papilla and the epithelial matrix in these follicles have been described in detail by Van Scott and Ekel (1958). During the early stages of alopecia areata it is likely that most, if not all, the follicles pass through telogen. Eckert (1968) studied over a thousand exclamation mark hairs and states that they were invariably normal club hairs. However, Headington, Mitchell and Swanson (1981) reported that catagen/telogen is morphologically abnormal in alopecia areata and coined the term 'nanogen' to describe this. Apart from the relative infrequency with which they contained club hairs, we were unable to detect abnormalities in catagen and telogen follicles in early lesions although early catagen was poorly represented in our biopsies.

The finding of anagen follicles in established lesions, in numbers which approach normal proportions, indicates that re-entry into anagen takes place. However, anagen development is halted at a stage when the inner root sheath has assumed a conical shape and before the cortex has begun to keratinize. This stage is equivalent to anagen III (Fig. 8). Van Scott (1958) stated that growth is halted in anagen IV, but at that stage cortical keratinization has commenced, and this was not seen in any of the follicles we studied. The follicles must then return prematurely to telogen as it would be impossible for a mitotically active anagen follicle to persist in anagen III. This is supported by the finding of catagen follicles in established lesions. These truncated cycles probably continue repeatedly until the disease activity subsides. Evidence of cycling in active lesions also comes from the finding of multiple hair shafts within individual follicles during early regrowth.

The nature and site of the primary pathology within the hair follicle in alopecia areata are uncertain. The hair bulb is a complex structure in which rapidly dividing matrix cells differentiate to form the various layers of the inner root sheath and the hair shaft. Morphological and auto-radiographic studies (Kligman, 1959; Epstein & Maibach, 1967) have indicated that matrix cells in the lower hair bulb predominantly go to form the inner root sheath while matrix cells lining the upper half of the dermal papilla mainly give rise to the hair cortex (precortical matrix). In an electron microscopic study of alopecia areata (Messenger & Bleehen, 1984), lesional anagen follicles showed evidence of non-specific injury to matrix cells around the upper pole of the dermal papilla and to cells of the presumptive cortex, i.e. cells undergoing early cortical differentiation. Cell injury was not seen in the lower bulb matrix nor in other differentiating compartments such as the inner root sheath. Degenerative changes can also be seen in the suprapapillary matrix by light microscopy, an observation first made by Thies (1966). We have also recently found that cells in the precortical matrix and presumptive cortex express HLA-DR in over 65% of anagen follicles from lesional areas of alopecia areata (Messenger *et al.*, 1984). This phenomenon was largely restricted to the cortical region of the hair bulb with less than 20% of follicles displaying HLA-DR expression in other epithelial compartments. HLA-DR expression in normal follicles was confined to dendritic cells which were very sparse below the level of the arrector insertion. Such aberrant expression of HLA-DR has been described in a number of disorders in which cell-mediated immune reactions are implicated. Although we have yet to determine the cause of cell injury in alopecia areata, its restriction to the cortical region of the hair bulb, together with the relative restriction of HLA-DR expression to the same area, strongly suggest that this site is of fundamental importance in the pathogenesis and may indeed form the primary target for the disease process within the anagen follicle. This concept is given added weight because it allows the alterations in the hair cycle to be explained.

The anagen follicle responds to the initial insult by reverting to telogen where cortical differentiation is not taking place. Some follicles are able to survive for a time in anagen but the impairment of cortex formation leads to the production of a dystrophic hair. If the initial insult is severe enough to cause cortical injury, there will be a focal weakness or narrowing in the hair shaft which may then fracture at that point when it reaches the skin surface. This event precedes telogen transformation or dystrophic anagen giving rise to the exclamation mark hair. The continued production of hair shaft after shaft fracture or narrowing, even in follicles undergoing telogen transformation directly, shows that alopecia areata does not cause an abrupt cessation of mitotic activity in the matrix. Eckert *et al.* (1968) suggested that the response to the initial insult was governed by disease severity but it is possible that other factors, such as duration in anagen, determine how individual anagen follicles respond. Once in telogen the follicle is 'safe'. Re-entry into anagen takes place normally, but as soon as cortical differentiation begins the attack is resumed, anagen development can proceed no further, and the follicle returns prematurely to telogen. Morphological evidence of cortical differentiation is first seen in anagen III. In the human hair bulb, melanin pigment is transferred exclusively to cortical keratinocytes and it is noteworthy that activation of hair bulb melanocytes also takes place in anagen III (Kukita, 1957). The ability of the hair follicle to revert to telogen when it comes under attack will explain why follicles are not permanently destroyed.

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# IgG subclasses in acne vulgaris

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## SUMMARY

IgG subclasses were measured in male patients with very low grade or severe acne. No IgG subclass deficiencies were found. Patients with severe acne had a significant increase in total IgG attributable to their exposure to antigens which stimulate the production of antibodies in the IgG2 and IgG3 subclasses.

Previous investigations have shown that in inflammatory acne there is an immunological response, particularly to the antigens of *Propionibacterium acnes* (Puhvel, Hoffman & Sternberg, 1966; Puhvel *et al.*, 1977; Gowland *et al.*, 1978; Kersey, Sussman & Dahl, 1980). Lymphocyte subpopulation studies in acne patients have suggested that this is a typical response to antigenic stimulation and that there is no regulatory defect of the host's immune system (Holland, Gowland & Cunliffe, 1983). However, normal serum IgG levels can mask an IgG subclass deficiency. Firstly, such an imbalance does not always manifest itself as a severe disturbance of humoral immunity and, secondly, it has been found that the lack of synthesis of one subclass may be compensated for by the increased synthesis of other subclasses (Oxelius, 1979). We have measured levels of the IgG subclasses and other immunoglobulins in acne patients to evaluate their immunological competence, and as a possible indicator of the type of antigen to which patients are responding.

## METHODS

Twenty-five male patients with severe acne (mean age 19·4 years; acne grades $>6·0$) and 38 control subjects with very low grade acne (mean age 19·5 years; acne grades $<0·8$) were studied. Male patients only were included owing to the higher frequencies of severe acne in males in comparison with females. Acne grades were assessed according to the method of Burke and Cunliffe (1984).

Serum concentrations of total IgG, IgM, IgA, IgG1, IgG2, IgG3 and IgG4 were measured by radial immunodiffusion (Mancini method). Radial immunodiffusion for IgG subclass estimations was performed in 1·4% agarose (Miles) in 0·1 M barbitone buffer pH 8·6 plus 6% PEG 4000, containing the following dilutions of monoclonal antibodies (Seward): 1·25% for IgG1 (BAM09); 1·0% for IgG2 (BAM010); 0·5% for IgG3 (BAM08); and for IgG4 0·25%

(BAM16). Test sera were diluted 1/6 for IgG1, IgG2 and IgG4 and 1/2 for IgG3, and incubations were carried out at 4°C. The World Health Organisation standard for immunoglobulins G, A and M, 67/86 was used as a standard reference serum (Rowe, Anderson & Grab, 1970).

## RESULTS

The serum immunoglobulin concentrations and the proportion of each IgG subclass expressed as a percentage of the total summated IgG are shown in Table 1. The concentrations of IgG2, IgG3, IgG4, IgM and IgA were not normally distributed. Comparisons between control subjects and patients with high grade acne were therefore analysed by the Wilcoxon rank sum test. Significantly higher levels of IgG2, IgG3, total summated IgG and total IgG were found in severe acne patients as compared with control subjects ($P < 0{\cdot}05$).

TABLE 1. Serum immunoglobulin and IgG subclass levels in control subjects and patients with severe acne

| | Control subjects | | | Severe grade acne patients | | |
|---|---|---|---|---|---|---|
| | Concentration (g/l) | | | Concentration (g/l) | | |
| Immunoglobulin | Median | Range | %* | Median | Range | %* |
| IgG1 | 6·54 | 4·14–8·9 | 62·00 | 6·60 | 3·81–11·95 | 57·5 |
| IgG2 | 2·88 | 0·37–6·12 | 27·90 | 3·48 | 1·80–6·60 | 31·03 |
| IgG3 | 0·44 | 0·19–1·89 | 4·97 | 0·61 | 0·12–1·82 | 5·76 |
| IgG4 | 0·44 | 0·04–1·62 | 4·97 | 0·52 | 0·04–2·56 | 5·64 |
| Total summated IgG | 10·48 | 6·51–15·90 | | 12·03 | 7·22–16·47 | |
| Total IgG | 8·87 | 4·77–11·71 | | 10·69 | 6·69–15·12 | |
| IgA | 1·47 | 0·69–4·76 | | 1·94 | 0·29–8·84 | |
| IgM | 0·96 | 0·48–2·64 | | 1·64 | 0·57–3·36 | |

* Expressed as a percentage of total summated IgG.

## DISCUSSION

Generally, antigens give rise to antibodies of all IgG subclasses, but certain antibody populations may be limited to a single IgG subclass. A direct correlation has been found between the serum IgG2 level and antibody response to polysaccharide and teichoic acid antigens (Silser *et al.*, 1980). Such antigens are present in Gram positive bacterial cell walls. The significant increase in total IgG in severe acne was due to significant increases in IgG2 and IgG3 subclasses, which suggests that a major part of the antigenic stimulation in acne is from Gram positive bacterial cell wall components. Possible organisms supplying these components are *Propionibacterium acnes* and, less probably, staphylococci and *Pityrosporum ovale*.

The present study confirms previous findings that there is an immune component to inflammatory acne and that patients with severe acne are immunologically competent. IgG subclasses were present in essentially similar proportions in patients with severe acne and control subjects and these proportions were in agreement with those found by French and Harrison (1984) in the sera of healthy adults, showing that there is no IgG subclass deficiency in acne.

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***Pharmacology and Treatment***

# Evaluation of a therapeutic strategy for the treatment of acne vulgaris with conventional therapy

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## SUMMARY

Four hundred and twenty patients with moderate to severe acne vulgaris were treated with oral erythromycin and topical benzoyl peroxide to determine the optimum dose regimes. Our results show that the response was significantly less in patients with a greater severity of acne, with truncal acne and in those with a higher sebum excretion rate. There was a significantly better clinical result in patients given 1g erythromycin daily than in those given 0·5 g daily (plus topical therapy in both groups). The relapse rate on stopping antibiotics is also significantly less in patients given 1 g daily and this dosage did not produce any increase in side effects. We suggest that any patient requiring oral antibiotics should initially be given 1 g daily of erythromycin (or tetracycline) for up to six months, plus topical therapy.

Acne is a common skin disorder, yet there are no clear guidelines as to how it should be treated. Many primary care practitioners and dermatologists have their favoured regimes. Some patients have been on antibiotics for years. Over the years we have evolved our own basic therapeutic regime (Gould & Cunliffe, 1978) using oral erythromycin 0·5 g daily and benzoyl peroxide gel 5% topically, both together for a six month period. We use erythromycin in preference to tetracycline only because much of our earlier clinical work on acne was centred on the use of this drug. However, controlled studies comparing the two antibiotics are lacking. There are many papers demonstrating the efficacy of benzoyl peroxide in mild to moderate acne (Kirton & Wilkinson, 1970; Liddell, 1974; Hare, 1975; Burke & Cunliffe, 1984).

The aim of this study was to determine whether the initial severity of the acne, the site of the acne or the sebum excretion rate influenced therapeutic response. We also compared the effects of 0·5 g and 1 g of erythromycin daily to determine whether there was a dose-related response.

## METHODS

Four hundred and twenty patients (170 males and 250 females) entered the study. All had

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moderate or severe acne and the age range was 11–38 years (mean age for males 18·5 years and for females 19·6 years). No patient had been on any acne therapy for 4–8 weeks before starting our treatment. All patients were instructed to take oral therapy half an hour before food, not with milk, and to apply the gel to all areas, not just to the lesions. The patients were seen at a control visit 1–3 weeks before starting treatment and then at two or three monthly intervals up to the end of the 4–6 month treatment period. On each occasion the acne severity was graded by the Leeds technique (Burke & Cunliffe, 1984), and sebum excretion rate was measured by the gravimetric technique (Strauss & Pochi, 1961). In the following studies some of the patients' results were incorporated into two or more sets of data. In all studies the doctors did not have access to the records of the initial acne score when the patient attended for follow-up.

1. In order to determine whether acne severity influenced response to treatment, two groups of 20 patients with facial acne, matched for age and sex, were given erythromycin 250 mg b.d. and 5% benzoyl peroxide for 4 months. One group had moderate, the other severe, acne (mean grades ± SEM, 2·25 ± 0·40 and 4·91 ± 0·71 respectively).

2. Eighty patients were assessed over a 6 month period to determine if, when given 250 mg erythromycin twice daily and 5% benzoyl peroxide, there was any site variation in their response.

3. The possible effect of sebum excretion was determined in two groups of 20 patients, matched for age, sex and acne severity (grade 3·21 ± 0·9) but differing in their sebum excretion rate; both groups were given 250 mg erythromycin twice daily and 5% benzoyl peroxide for 6 months.

4. Two groups of 148 patients were given either 0·5 g or 1·0 g erythromycin daily and 5% benzoyl peroxide for 6 months and side effects assessed.

5. Two sets of 76 patients matched for age, sex, site of involvement and acne severity (grades 3·31 ± 0·29 and 3·19 ± 0·34) were given either 0·5 g or 1·0 g erythromycin daily and 5% benzoyl peroxide for 6 months and the rate of improvement noted. In this group, after 6 months the antibiotic was withdrawn over a 3 week period and the patients were told to continue with topical treatment. These patients were followed up for a further 12 months.

Statistical analyses were carried out using analysis of co-variance, the Wilcoxon test, the Mann-U Whitney test and Student's *t*-test.

TABLE 1. Clinical response in patients with severe and moderately severe acne given 0·5 g erythromycin and topical 5% benzoyl peroxide

| | Initial acne grade (mean ± SEM) | Acne grade at 4 months (mean ± SEM) | Percentage improvement |
|---|---|---|---|
| Severe acne | 4·91 ± 0·71 | 3·88 ± 0·52* | 21 |
| Moderate acne | 2·25 ± 0·40 | 1·35 ± 0·29** | 45† |

Statistical analysis using Mann-U Whitney test.
* Comparison with initial acne grade $P < 0{\cdot}05$.
** Comparison with initial acne grade $P < 0{\cdot}001$.
† Compared with percentage improvement in severe acne $P < 0{\cdot}05$.

TABLE 2. Response of acne at different body sites in patients receiving 0·5 g erythromycin and 5% benzoyl peroxide

| Sex | Site | Initial acne grade | Acne grade at 3 months (% improvement) | P* | Acne grade at 6 months (% improvement) | P |
|---|---|---|---|---|---|---|
| Female | face | 3·61 | 2·16 (40%) | NS | 1·16 (68%) | <0·05 |
| Female | back | 1·62 | 0·97 (40%) | | 0·78 (52%) | |
| Male | face | 4·11 | 2·55 (38%) | <0·05 | 1·44 (65%) | <0·05 |
| Male | back | 1·84 | 1·34 (27%) | | 0·90 (51%) | |

* Comparison between groups by Mann-U Whitney test.

## RESULTS

1. Acne severity was found to influence the therapeutic response; patients with severe acne responded significantly less well than those with moderate acne when given identical treatment (Table 1).

2. Independent of overall acne severity, truncal acne had responded significantly less well than facial acne in both sexes after 6 months of treatment (Table 2). After 3 months of treatment there was a significant difference between the responses of truncal and of facial acne in males but not in females.

3. Sebum excretion rate (SER) was found to influence the rate of response. In both sexes, patients with a high SER showed a slower rate of response. There was a significant difference in response after 6 months of treatment in both sexes (Table 3), and in males after 3 months.

4. Doubling the dose of erythromycin from 0·5 g daily to 1·0 g daily did not increase the incidence of side-effects (Table 4).

TABLE 3. Response of acne patients given 0·5 g erythromycin and 5% benzoyl peroxide in relation to SER

| | | Mean % improvement in acne grade | | |
|---|---|---|---|---|
| | | Months of treatment | | |
| Sex | SER | 1 | 3 | 6 |
| Female | high (mean 1·92 $\mu g/cm^2$) | 27 | 40 | 51 |
| Female | low (mean 0·81 $\mu g/cm^2$) | 28 | 58 | 70* |
| Male | high (mean 2·23 $\mu g/cm^2$) | 29 | 42 | 53 |
| Male | low (mean 0·92 $\mu g/cm^2$) | 25 | 56* | 67* |

* Comparison with high SER group; analysis of covariance $P<0·005$.

TABLE 4. Incidence of side-effects in patients taking 1 g or 0·5 g erythromycin daily

| Dose of antibiotic/day | 1 g | 0·5 g |
|---|---|---|
| Number of patients | 148 | 148 |
| Number of patients suffering gastro-intestinal side-effects (colic with or without diarrhoea) | 14 | 12 |
| Vaginal candidiasis | 1 | 1 |

TABLE 5. Comparison of the clinical improvement in patients receiving either 0·5 g or 1·0 g erythromycin plus topical benzoyl peroxide

| | | | Mean % improvement in acne grade | | | % of patients relapsing within 12 months after ending oral treatment |
|---|---|---|---|---|---|---|
| Sex | No. of patients | Dosage | 2 months | 4 months | 6 months | |
| F | 39 | 0·5 g | 43·5 | 51·5 | 58·7 | 60 |
| F | 39 | 1·0 g | 41·5 | 59·9 | 78·7* | 31* |
| M | 40 | 0·5 g | 23·0 | 28·7 | 35·2 | 82 |
| M | 38 | 1·0 g | 39·2** | 59·8* | 78·4* | 39* |

Comparison with patients receiving 0·5 g, Mann-U Whitney test.
* $P<0{\cdot}001$.
** $P<0{\cdot}01$

5. Doubling the dose of the antibiotic significantly increased the response in males after 2, 4 and 6 months and in females after 6 months (Table 5).

In the group receiving 0·5 g erythromycin daily, the relapse rate (defined as a return of the acne to 50% or more of the original severity) was 82% in males and 60% in females, within one year of stopping antibiotic therapy. In the group receiving 1 g daily 39% of males and 31% of females had relapsed within one year. Although males fared less well than females in both regimes there was no statistically significant difference between the sexes (Table 5).

## DISCUSSION

Over the past 20 years doctors must have treated millions of patients for acne with oral antibiotics and topical treatments such as benzoyl peroxide and retinoic acid. The results must have varied enormously since there is little data to guide the physician on the choice of therapy.

Our study has shown conclusively that if oral erythromycin or tetracycline are to be used, a dose of 1 g daily significantly improves the response with no increase in side-effects. A dose-related response is in good agreement with a previous study which demonstrated the effectiveness of large doses of tetracycline in patients with severe acne, especially those who had failed to respond to small doses (Cunliffe *et al.*, 1973; Cunliffe *et al.*, 1981; Baer, Leshaw & Shalita, 1976). We have also previously shown that females responded better than males when given 0·5 g of erythromycin daily (Cunliffe *et al.*, 1981) and we suggested that this was because females have a lower SER than males. Our present study supports this view in that patients with a high SER responded significantly less well than those with a low SER. It is possible that a higher concentration of drug is achieved in the pilosebaceous duct in patients with a low SER because of a reduced 'wash out' effect. The higher dose also overcame the sex-related difference in response, suggesting that it produces an optimum concentration in the sebum in both sexes.

It was also apparent that on 0·5 g erythromycin truncal acne responded less well than facial acne, perhaps because of the more careful application of benzoyl peroxide to the face. This is unlikely to be the whole explanation, however, as truncal acne also responds less well to oral 13-*cis*-retinoic acid (Jones *et al.*, 1983). There is probably some difference in the way drugs reach the different skin sites and are subsequently metabolized.

We have also demonstrated the benefit of 1 g of erythromycin daily in reducing the rate of relapse. At the end of six months we tell the patient to reduce the antibiotic by one tablet weekly. We do not know if this is better than sudden cessation of oral therapy but it seems more acceptable to the patient. We also instruct the patient to continue with topical therapy, since we have shown that acne relapses after stopping topical treatment (Cunliffe *et al.*, 1981). Why the higher dose of antibiotic is clinically superior to 0·5 g daily is not entirely clear. Oral antibiotics reduce surface *P. acnes* but only by up to 10-fold (Leyden, McGinley & Kligman, 1982). They also influence the metabolic activity of *P. acnes* (Marples, Downing & Kligman, 1970), including the production of chemoattractant agents (Esterly *et al.*, 1984), and indirectly influence ductal cornification (Holmer, Williams & Cunliffe, 1972). However, adequate *in vivo* dose response studies of these variables are lacking. Comparison of separate, and therefore not strictly comparable, studies, suggests that there is a dose-related effect in that 250 mg daily of tetracycline showed no effect on surface *P. acnes* whereas 1 g daily of tetracycline produces a statistically significant 10-fold reduction.

We decided to use topical and oral treatment in the present study to mirror the regime used in the clinics. This decision has been supported subsequently by the preliminary analysis of some ongoing work in our clinics which indicates that topical treatment does have an additional and significant clinical benefit over and above oral therapy. Many questions still remain unanswered. For example, would we get a lower rate of relapse if we treated for 8 or 10 months rather than 6 months? Although we advise our patients to take the tablets in a dosage of 250 mg four times a day, would 500 mg twice daily be equally successful? Should topical therapy be used once or twice daily? We wish to emphasise that we used erythromycin because we had used it in previous studies (Gould & Cunliffe, 1978) and so use of the same drug made comparisons easier.

As a result of this study we have changed our therapeutic regime and recommend this regime to other colleagues. We now start all patients for whom antibiotics are required on 1 g of erythromycin (or tetracycline) daily and continue this for 6 months, using benzoyl peroxide 5% (or, if appropriate, retinoic acid) topically. At the end of 6 months the oral antibiotic is gradually withdrawn over a 3 week period but topical therapy should be continued.

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***Case Reports***

# Neurological complications in patients with cutaneous T-cell lymphoma

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## SUMMARY

Five patients with a cutaneous T-cell lymphoma (four with mycosis fungoides and one with a primary cutaneous T-immunoblastic lymphoma) who developed neurological sequelae are reported. In four of them, central nervous system involvement could be demonstrated at autopsy. The importance of early cerebrospinal fluid examination in these patients is stressed.

Reports of cental nervous system (CNS) involvement in cutaneous T-cell lymphoma (CTCL), which include mycosis fungoides (MF), Sézary's syndrome and primary cutaneous T-immunoblastic lymphoma (T-IBL), are rare, though it is well documented in other non-Hodgkin's lymphomas (Levitt *et al.*, 1980; MacKintosh *et al.*, 1982). The various casuistic reports have recently been reviewed by Bodensteiner and Skikne (1982) and Zackheim *et al.* (1983).

We report five patients with a CTCL and neurological symptoms, four of whom were found to have CNS involvement at autopsy. The importance of early neurological examinations in CTCL patients suspected of having CNS involvement, and some therapeutic possibilities, are discussed.

## PATIENTS

In our clinic, between 1974 and 1984, five patients (two females and three males, ages 61–77 years) were seen with neurological sequelae of a CTCL (Table 1). Four patients had MF, tumour stage, and one had a primary cutaneous T-IBL. The criteria on which the diagnosis of MF was made, and the stages distinguished, have been published previously (Hamminga *et al.*, 1982). The diagnosis of T-IBL was based on the results of histological examination of skin biopsies, marker studies and enzyme histochemistry.

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TABLE 1. Clinical data and autopsy findings in

| No. | Patients: Age (years) | Sex | Lymphoma type | stage* | Cytostatic therapy | Presenting neurological symptoms | Neurological diagnosis | CSF examination |
|---|---|---|---|---|---|---|---|---|
| 1 | 64 | M | MF | IIIc | CVP | Pain left leg; 3 weeks later in both legs; difficulties in walking | Cauda equina syndrome | WBC 900/$\text{mm}^3$<br>protein 8·0 g/l<br>cytological class IV† |
| 2** | 77 | F | MF | Ic | — | Difficulties in walking; 6 weeks later speech difficulties | Cerebellar dysarthria and ataxia; spastic paraparesis | WBC 2/$\text{mm}^3$<br>protein 0·89 g/l<br>cytology not abnormal |
| 3 | 61 | M | MF | IIIc | V, C, Proc. | Dizziness; 3 weeks later left sided weakness | Slight dementia and paraparesis | WBC 1/$\text{mm}^3$<br>protein 0·59 g/l<br>cytology not abnormal |
| 4 | 74 | F | MF | IIId | CVP | — | Stupor | ND |
| 5 | 70 | M | T-IBL | Ic | CVP<br>CHVP<br>Bleo<br>VM 26 | Difficulties in walking; memory disturbance | Cerebellar ataxia; generalized convulsions; impaired consciousness | WBC 400/$\text{mm}^3$<br>protein 1·29 g/l<br>cytological class V† |

MF: mycosis fungoides;
T-IBL: T-immunoblastic lymphoma;
*I: confined to the skin;
III: with lymph node involvement;
c: tumours;
d: erythroderma;
Classification according to the Dutch mycosis fungoides study group (van Vloten, 1982).
CVP: cyclophosphamide, vincristine and prednisone;

patients with CTCL and neurological sequelae

| CT-scan | Autopsy findings | Survival after nervous system involvement |
|---|---|---|
| Diffuse cerebal atrophy | Infiltration in meninges of cerebrum, cerebellum; extension in anterior and posterior roots; perivascular infiltration of cerebral parenchyma; bilateral inguinal lymph node involvement. No involvement of viscera | 17 weeks |
| | Perivascular infiltration in both cerebral hemispheres, thalamus, hypothalmus, cerebellum and spinal cord with demyelinization and motor neurone loss. No MF in viscera or lymph nodes | 12 weeks |
| Right-sided parietal hypodense mass lesion without enhancement | Progressive multifocal leukencephalopathy. No MF in viscera or lymph nodes | 8 weeks |
| ND | Infiltration in dura mater, in inguinal, parailiacal and paraaortal lymph nodes, in mediastinum and almost all visceral organs | 4 days (after development of stupor) |
| Contrast enhancement vermis cerebelli | Extensive infiltration in subarachnoidal space and perivascular areas in cerebrum, cerebellum and medulla oblongata. Also diffuse infiltration in cerebral parenchyma. No visceral or lymph node involvement | 4 weeks |

V: vincristine;
C: cyclophosphamide;
Proc.: procarbazin;
CHVP: cyclophosphamide, adriamycin, vincristine and prednisone;
Bleo: bleomycin;
VM 26: teniposide;
† Cytological classification according to Papanicolaou;
ND: not done;
** This patient has been described previously by van Erp *et al.* (1981).

Four patients had received polychemotherapy prior to the development of neurological symptoms. In four patients some minor neurological disturbances, which varied from difficulties in walking and dizziness to speech and memory disturbances, preceded the full neurological picture by some weeks. At that time three patients had either an abnormal CT-scan of the brain or a pathological CSF cytology (Table 1).

Four patients showed infiltration of the CNS by neoplastic cells at autopsy; one patient (Case 3) had no direct CNS involvement but had a progressive multifocal leukencephalopathy, a disorder which may complicate lymphoproliferative diseases. Two patients (Cases 1 and 2) showed meningeal as well as perivascular infiltration of the parenchyma of the brain, whereas Case 4 had only involvement of the dura mater. In three cases the infiltrates were predominantly composed of atypical lymphoid cells with convoluted or cerebriform nuclei, similar to those observed in the skin. In the patient with T-IBL (Case 5), autopsy showed infiltration in the subarachnoidal and perivascular spaces as well as extensive, diffuse infiltration of the lymphoma throughout the entire cerebral parenchyma with large numbers of blast cells having round or lobulated nuclei with one or several prominent nucleoli. Similar cells had been present in all previous skin biopsies. Immunohistochemical studies of skin and brain tissue showed that these cells had the phenotype of transformed T-cells (Leu-$1^{-/+}$, $2a^-$, $3a^+$, $4^+$, HLA-$DR^-$, B-$1^-$).

The clinical course of Case 1 is presented in more detail, as it clearly illustrates the need for early diagnosis and treatment of CNS involvement in patients with CTCL.

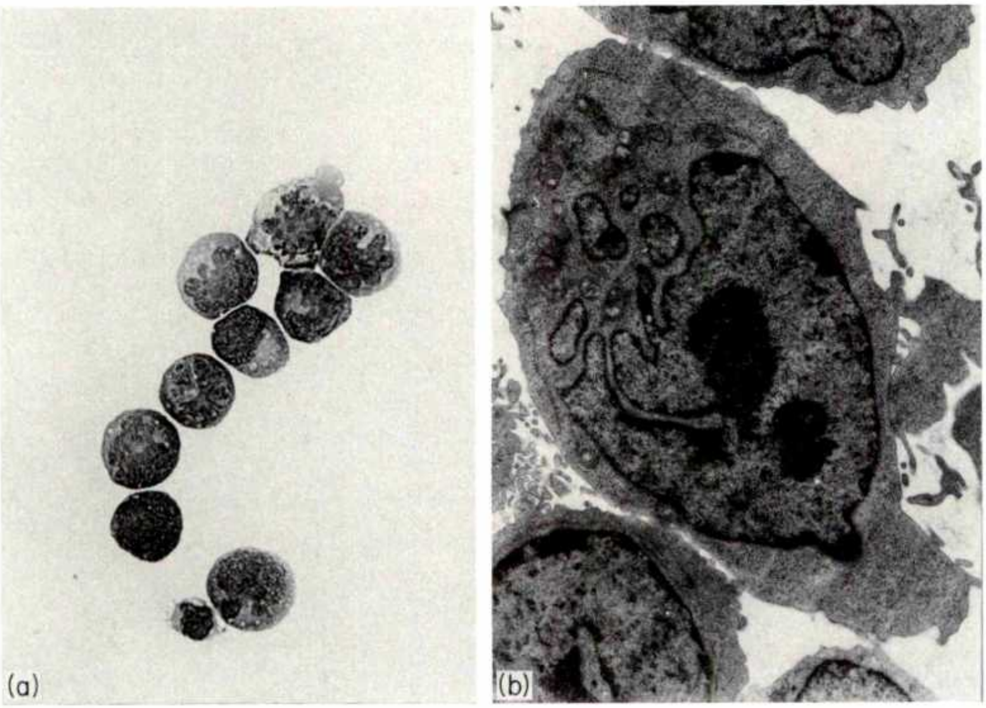

FIGURE 1. (a) Pleiomorphic mononuclear cells with cerebriform lobulated nuclei in the cerebrospinal fluid. (Case 1) (May-Grünwald-Giemsa stain × 750); (b) cell with cerebriform nucleus in CSF (Case 1) (Original × 7500).

## CASE REPORT

A 66-year-old male patient was referred to the Department of Dermatology with tumour stage MF, two years after the first skin lesions appeared. Physical examination and laboratory investigations revealed no lymph node or visceral involvement. He was treated with total-skin electron beam irradiation (4 MeV, 35 Gy) for 10 weeks with an additional irradiation of 10 Gy on the tumours, which resulted in a complete remission. One year later lymph node involvement was confirmed histologically in enlarged inguinal lymph nodes. Treatment was started with a polycytostatic regimen consisting of 3-weekly courses of cyclophosphamide (300 mg orally for five days), vincristine (2 mg i.v. on day 1) and prednisone (40 mg orally for five days) (CVP). Although the lymph nodes decreased in size, new tumours developed during this treatment. After five CVP courses the patient experienced radicular pains, initially in his left leg and three weeks later in both legs, associated with difficulties in walking and standing. Two hours before his admission to the Department of Neurology he had a generalized seizure. Physical examination revealed a paraparesis of his legs, absent knee and ankle jerks with an extensor plantar response on the right and a flexor response on the left. Sensory examination was abnormal for all modalities in both his legs.

CSF examination showed a pleiocytosis (WBC 900/mm$^3$), an elevated protein concentration (8·0 g/l) and a low glucose level (0·7 mmol/l). Electrophoresis revealed a transudation pattern and cytology showed the presence of large mononuclear cells with vesicular or lobulated nuclei (Figs 1a and 1b). Immunofluorescence studies on CSF using monoclonal antibodies showed that the large majority of the neoplastic cells were reactive with pan-T cell antiserum OKT3. The percentages of OKT4$^+$, OKT8$^+$ and OKT11$^+$ cells were 7, 4 and 8 respectively, indicating that these malignant T-cells had lost several differentiation antigens present on normal T-lymphocytes. Lumbar myelography suggested meningeal infiltration with obliteration of the root pockets. The electroencephalogram was diffusely abnormal. The CT-scan of the brain showed diffuse cerebral atrophy, and the persistence of contrast material in the ventricles after myelography suggested reversal of CSF flow.

The clinical diagnosis was lymphomatous leptomeningitis and cauda equina syndrome. Five

TABLE 2. Cerebrospinal fluid examination and the effect of intrathecal methotrexate therapy in Case 1

| | Time after starting methotrexate on diagnosis of CNS involvement | | | | | | |
|---|---|---|---|---|---|---|---|
| | Days | | | Weeks | | | |
| | 0 | 2 | 4 | 1 | 2 | 3 | 17 |
| Methotrexate given | + | + | + | + | + | – | – |
| WBC cells/mm$^3$ (normal $<3$) | 900 | 33 | 52 | 20 | 4 | 5 | 410 |
| Protein g/l (normal $<0{\cdot}45$) | 8·04 | – | 2·24 | 1·56 | 1·34 | 0·99 | 13·8 |
| Glucose mmol/l | 0·7 | – | 3·9 | 4·1 | 4·6 | 3·8 | 2·1 |
| Cytological class (Papanicolaou) | IV | IV | III | III | II | I | IV |

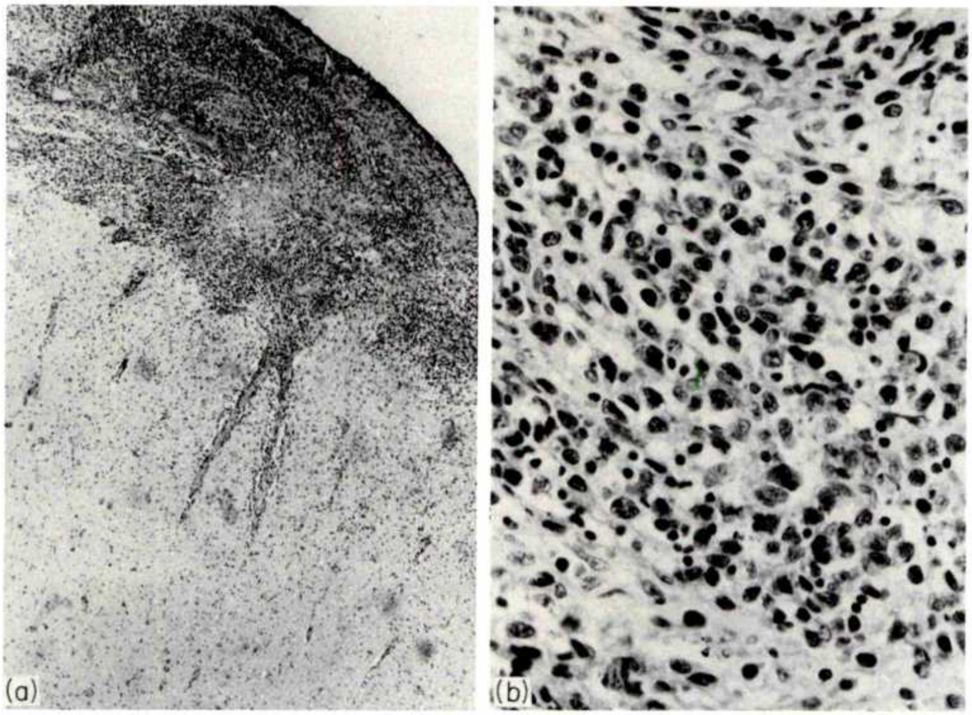

FIGURE 2. (a) infiltration from the arachnoidal space via the perivascular space into the underlying parenchyma of the brain (Case 1) (H & E × 50); (b) atypical lymphoid cells with lobulated or cerebriform nuclei, mixed with small lymphocytes infiltrating the parenchyma. A few mitotic figures are present (Case 1) (H & E × 500).

courses of 15 mg methotrexate intrathecally resulted in a striking reduction of CSF cells and an improved cytological score (Table 2). The motor function of his legs gradually improved. However, one month later, the cutaneous and lymph node involvement progressed and three additional CVP courses were given. In the following weeks a bilateral foot-extensor paralysis developed in addition to a frank psychosis with paranoid hallucinations. CSF examination suggested progression of the CNS involvement (Table 2), but further intrathecal therapy was refused by the patient. In the terminal stages he developed progessive dysphagia, dysarthria, amimia, tachycardia and tachypnoea. He died 17 weeks after demonstration of CNS involvement.

At autopsy bilateral pneumonia and a pulmonary embolus were found. Apart from bilateral inguinal lymph node involvement no other signs of MF could be found in the visceral organs. The brain was oedematous, weighing 1330 g, and at several places the surface displayed a grey-reddish discoloration together with thickening of the arachnoid. Histopathological examination revealed infiltration of the arachnoidal space by a large number of atypical lymphoid cells, including blast cells, with lobulated or cerebriform nuclei. In many of these cells mitotic figures were present. These cells were mixed with lymphocytes and a few dispersed plasma cells. From the arachnoidal space via the perivascular spaces there was infiltration of neighbouring parenchyma (Figs. 2a and b). In these areas thrombosis and haemorrhages were also observed. The more central parts of the brain parenchyma were free from tumour cell infiltration.

## DISCUSSION

As may be concluded from the sparse reports in literature, CNS involvement in patients with MF is an infrequent complication. Yet meningeal infiltration has been reported in 9% and 14% respectively of MF patients coming to autopsy in two large series (Epstein *et al.*, 1972; Rappaport & Thomas, 1974), suggesting that CNS involvement is not quite so rare as previously thought. This discrepancy is partly explained by the fact that the presenting neurological symptoms may be minor and may not be easily recognized. Moreover, there exists a great variety of presenting neurological symptoms, which is in keeping with the various CNS localizations found at autopsy. Meningeal as well as perivascular or diffuse involvement of the brain parenchyma have been observed at autopsy (Zackheim *et al.*, 1983). Whereas in other non-Hodgkin's lymphomas, bone marrow involvement appears to be a risk factor for CNS problems (Young *et al.*, 1979; Levitt *et al.*, 1980), in CTCL the severity of the skin disease alone (i.e. the presence of tumours and erythroderma) is related to the CNS complications (Zackheim *et al.*, 1983).

Since 1974 we have seen five patients with a CTCL, who had neurological sequelae of their lymphoma. Four of these patients had advanced MF and one patient had a primary cutaneous T-IBL. In most cases, T-IBL of the skin develops from a pre-existing MF or Sézary's syndrome (Goos & Cristophers, 1982). However, in our patient the clinical and histological manifestations were, from the outset, inconsistent with the diagnosis MF. The development of CNS involvement in a patient with a primary cutaneous T-IBL without lymph node or visceral involvement has not been reported previously.

CSF examination was performed in four patients and provided convincing evidence for CNS involvement in two of them (Cases 1 and 5). In their review of patients with MF and CNS involvement, Zackheim *et al.* (1983) found that CSF examination was positive in 9 out of 11 patients; therefore this may be regarded as a highly diagnostic procedure. Retrospective analysis of our patients revealed, in four of them, that minor neurological disturbances were present some weeks before the possibility of CNS involvement was considered and neurological examinations were performed. This rare complication might be treated more effectively if recognized and diagnosed at an earlier stage.

CNS involvement in patients with a CTCL can be treated with intrathecal chemotherapy, such as methotrexate (MTX), whole-brain irradiation or a combination of these. In MF, good responses to intrathecal MTX have been described (Hauch *et al.*, 1975; Lundberg, Cadmen & Skeel, 1976; Bodensteiner & Skikne, 1982). One of our patients (Case 1) received intrathecal MTX therapy, which was effective until he refused further treatment.

It is not clear how CNS invasion actually takes place in these patients. Perivascular space involvement, which was found to be the most prominent histological feature of CNS involvement (Zackheim *et al.*, 1983), was observed at autopsy in three out of four patients with CNS involvement. The histological findings in Case 1 illustrate that CNS involvement in MF may start in the arachnoidal space and may expand from there via the perivascular spaces into the neighbouring cerebral parenchyma.

While CNS involvement in patients with a CTCL is a rare complication, should early and aggressive cytostatic therapy of the CTCL result in prolonged survival, then the likelihood of CNS involvement might increase. This is conceivable since the therapeutic agents do not cross the blood-brain barrier and leave latent localizations in the CNS unattacked. Therefore regular neurological examination of all CTCL patients is advised, including CSF examination, should any neurological symptoms or signs appear. This approach offers the best opportunity for diagnosis of early CNS involvement, and therefore the possibility of early intrathecal treatment, in a group of patients whose prognosis is still very poor.

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# Pachyonychia congenita Jackson-Lawler type: a distinct malformation syndrome

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## SUMMARY

A family with three members in two generations affected by pachyonychia congenita, hyperkeratosis and hyperhidrosis of the palms and soles, follicular keratosis, neonatal teeth and epidermoid cysts (Jackson-Lawler syndrome) is described.

The nosological autonomy of this condition is proposed and a further heterogeneity is suggested on the basis of histopathological changes in the subcutaneous cysts.

Pachyonychia congenita is a genetically determined ectodermal dysplasia inherited as an autosomal dominant trait. On the basis of phenotype analyses, Gorlin *et al.* (1976) proposed that two distinct entities could be recognized: the more frequent Jadassohn-Lewandowsky syndrome characterized by pachyonychia congenita, hyperkeratosis and hyperhidrosis of the palms and soles, follicular keratosis and oral leukoplakia, and the less frequent Jackson-Lawler syndrome (Jackson & Lawler, 1951), characterized by neonatal teeth and epidermoid cysts in addition to the same nail and skin abnormalities, but no oral leukokeratosis; both forms have never been observed in the same family.

We report a family in which three members in two generations were affected by the rarer form of pachyonychia congenita, and describe in detail the clinical picture of this rare malformation syndrome.

## CASE REPORT

### *Case 1*

The proband, a 21-year-old female and the second child of unrelated 39-year-old parents, was born at term following an uneventful pregnancy and delivery. Birth weight was 3·8 kg (90th percentile), and neonatal teeth were present. At one month of age, nail dystrophy of hands and feet was noted. Armpit folliculitis, and cysts in the metacarpal and elbow regions were observed at puberty. Growth and psychomotor development were normal, and scholastic performance was good.

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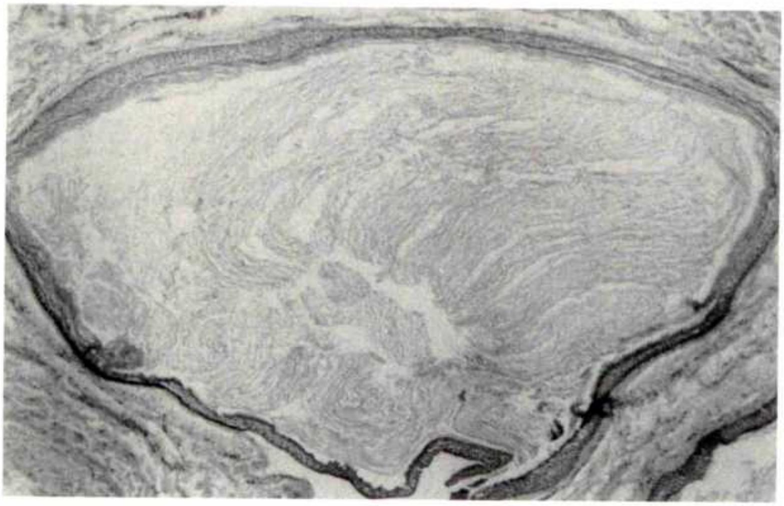

FIGURE 1. Epidermoid cyst with encysted lamellated keratin. Several cellular layers may be seen in the epidermal wall. (Haematoxylin-Eosin, × 60).

When she came under our observation, her weight was 56 kg (25th-50th percentile), her height was 160 cm (25th-50th percentile), and head circumference was 53 cm (10-25th percentile). Her hair was dry, bristly and sparse in the parietotemporal region; the eyebrows were very sparse. Except for malaligned teeth there were no oral abnormalities; she had a hoarse voice. Subcutaneous nodules adhering to the overlying normal skin were present on the left elbow (two nodules, 1 cm and 2 mm in diameter), on the dorsal surface of the proximal phalanges of the 3rd and 4th fingers of the left hand, and 2nd and 4th fingers of the right hand (several, 1-2 mm), and on the right knee (several, 1-2 mm). Follicular dermatosis was localized mainly on the back, armpits and pubic region. Palmoplantar hyperkeratosis with multiple foci particularly evident at the pressure points, and marked palmoplantar hyperhidrosis were also present.

The opaque, thickened nails of both hands and feet curved upwards, and were formed by yellowish grooved laminae; subungual keratosis was evident. Corneal dystrophy was not observed and fundus oculi was normal.

Histological examination of the elbow skin cyst disclosed a unilocular epidermoid cyst with encysted lamellated keratin (Fig. 1).

### *Case 2*

The proband's mother was a 61-year-old housewife. She had had neonatal teeth, and nail dystrophy was observed during the first year of life. At our examination her weight was 61 kg, height was 165 cm, and head circumference was 54 cm (50th percentile). Her hair was thin and dry, and eyebrows were absent. She had no dysmorphic facial features nor oral or ocular abnormalities; her teeth were malaligned, but normal. Her voice was hoarse.

Several subcutaneous nodes and nodules adhered to the overlying normal skin on the elbows (from 0·5 to 2·5 cm in diameter), on the dorsal surface of the proximal phalanges of the 2nd and 3rd fingers of the right hand and the 1st and 2nd fingers of the left hand (1-2 mm), on the middle phalanx of the 3rd finger of the left hand (3 mm) (Fig. 2), on the knees and on the pretibial regions (0·5-2·5 cm).

Pustular folliculitis was present, mainly in the armpit and pubic regions; palmoplantar hyperkeratosis with multiple foci associated with marked palmoplantar hyperhidrosis were also

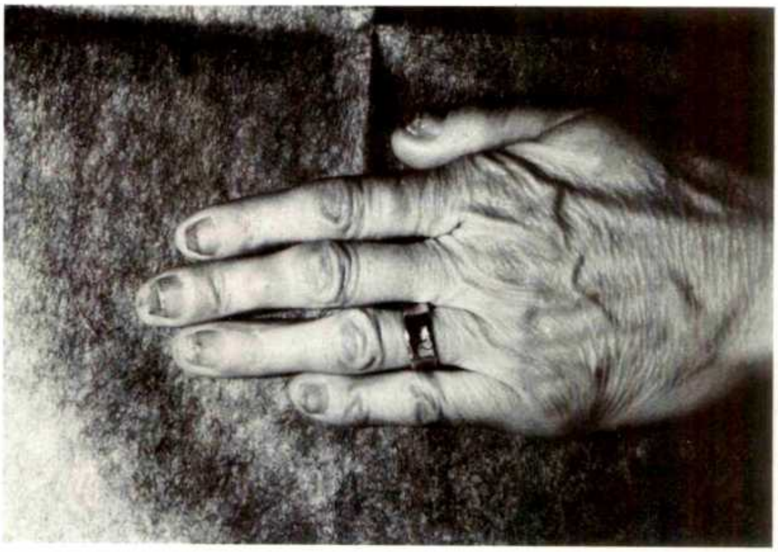

FIGURE 2. Left hand of Case 2. Nails are opaque and thickened; nodules are evident on the dorsal surface of 1st, 2nd and 3rd fingers.

observed. The nails of hands and feet showed the same changes seen in the proband. Microscopic examination of one elbow cyst revealed a unilocular epidermoid cyst with encysted lamellar keratin.

### *Case 3*

The proband's sister had died thirty years previously at six months of age from pneumonia. She is reported to have had nail dystrophy since birth and neonatal teeth, but no skin cysts were observed.

## DISCUSSION

Gorlin *et al.* (1976) distinguished two different patterns of pachyonychia congenita; they called the association of pachyonychia congenita with palmoplantar hyperkeratosis and hyperhidrosis, follicular keratosis, neonatal teeth and subcutaneous cysts the Jackson-Lawler syndrome. However, McKusick (1983) in his catalogue, stressed that the genetic relationship between the two syndromes (no. 16720; no. 16721) is unclear. Goodman and Gorlin (1983) described the two syndromes as distinct, but they were doubtful regarding their legitimate separation; Smith (1982) considers the two syndromes as one entity.

Eleven families with one or more members affected by the Jackson-Lawler syndrome are described in the literature; members of the original family reported by Jackson and Lawler (1951) have also been described by Shrank (1966), and Besser & Moynahan (1971). The condition is inherited as an autosomal dominant trait with complete penetrance and variability of expression; only two sporadic cases have been reported (de Groot, 1966; Moldenhauer & Seidel, 1973). In the family we describe, three members in two generations were affected: the proband and her mother with full expressivity, while only the incomplete syndrome was present in the sister; although it must be emphasized that expressivity is, in part, age-dependent, i.e., skin cysts and keratosis are more evident in older patients. In the nine families described, more than 50 affected members are reported, but in only a proportion of these were clinical details included. The most frequent clinical findings are thickened nails, cutaneous cysts, palmoplantar hyperkeratosis and hyperhidrosis, neonatal teeth, and dry thin hair. Other anomalies such as a hoarse voice and corneal dystrophy are less frequently described; leukokeratosis of mouth and tongue has not been reported previously.

Histological examination of the subcutaneous cysts has disclosed epidermoid cysts in three previous cases (Soderquist & Reed, 1968; Besser & Moynahan, 1971; Boxley & Wilkinson, 1971) and in two of three affected members of the family we describe; steatocystoma multiplex was detected in 6 cases (Vineyard & Scott, 1961; de Groot, 1966; Engel & Pinzer, 1966; Ionescu, Wolfshaut & Cernaianu, 1968; Moldenhaver & Seidel, 1973; Hodes & Noris, 1977). In two families no histological examination of the cutaneous cysts was performed (Sertoli, 1949; Schonfeld, 1980).

The concomitant presence of ectodermal dysplasia and subcutaneous cysts, with no mucous membrane involvement in probands or their affected relatives, strongly supports the hypothesis that a single autosomal dominant gene with complete penetrance and age-dependent expressivity is responsible for the pachyonychia congenita syndrome of the Jackson-Lawler type.

The histopathological difference in the skin cysts suggests a further genetic heterogeneity in this syndrome. For this reason, histological examination of subcutaneous cysts should be performed in all affected family members in order to ascertain whether histopathological heterogeneity between and within families exists.

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# Bone dependent nail formation

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## SUMMARY

Two sporadic cases with congenital anonychia and hypoplastic nails combined with ectrophalangia or hypoplastic phalanges are reported. It is suggested that congenital anonychia and hyponychia may be 'bone territory' dependent disorders.

Congenital absence of nails (anonychia) occurs sporadically and may exhibit either dominant or recessive inheritance (Juhlin & Baran, 1984; Tennstedt, Lachapelle & Baran, 1985; Ortonne, Juhlin & Lacour, 1985; Schot, 1978). Absent terminal phalanges are often associated with corresponding omission of the associated nail. Lack of phalanges (ectrophalangia) may arise in isolation or may be associated with other skeletal or dental abnormalities.

We report two sporadic cases of anonychia and hypoplastic nails associated with ectrophalangia or hypoplastic phalanges. They illustrate that a defect of the underlying bone may be a prerequisite for congenital anonychia and hyponychia.

## CASE REPORTS

### *Case 1*

This was a 23-year-old woman with total anonychia on her left 2nd finger and all her toes (Figs 1 & 2). The nails were rudimentary or hypoplastic on the 3rd and 4th digits of both hands. She was otherwise in good health and without other skeletal or dental abnormalities. X-ray examinations at the age of 2 months and 2 and 13 years had demonstrated absence of all distal and middle phalanges on the feet. The proximal phalanges were hypoplastic (Fig. 3). On the fingers, all the proximal phalanges were very slender distally and lacked a processus unguicularis (Fig. 4). She had no middle phalanx on the 2nd left finger. Calcification was normal for the ages examined.

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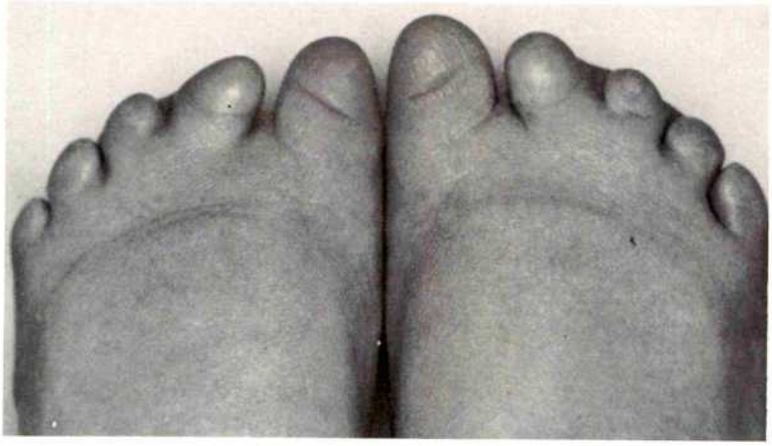

FIGURE 1. Anonychia of toes (Case 1).

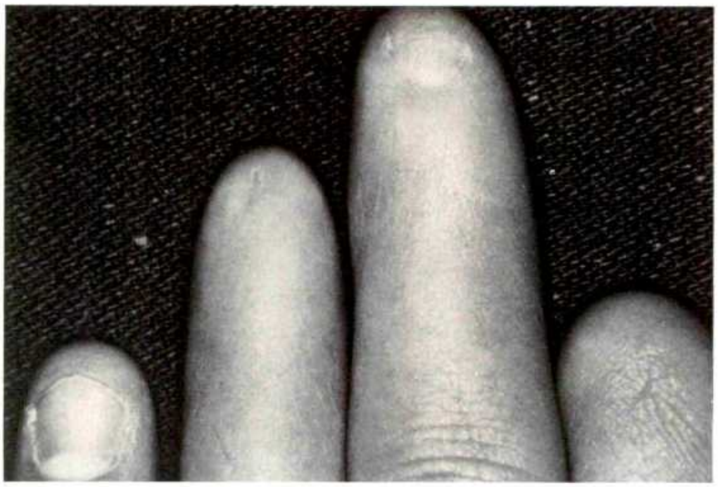

FIGURE 2. Anonychia of left digit II and hyponychia of digits III and IV (Case 1).

*Case 2*

An 82-year-old woman presented with brachydactyly of the 3rd and 4th fingers on her right hand. The nails were hypoplastic (Fig. 5). There had never been any significant functional defect. She now sought medical advice for the first time, purely for cosmetic reasons. Radiology revealed an absence of the middle phalanx of the 3rd digit and a vestigial middle phalanx on the 4th finger. Both distal and proximal phalanges on the 3rd and 4th fingers were hypoplastic but a processus unguicularis was present (Fig. 6).

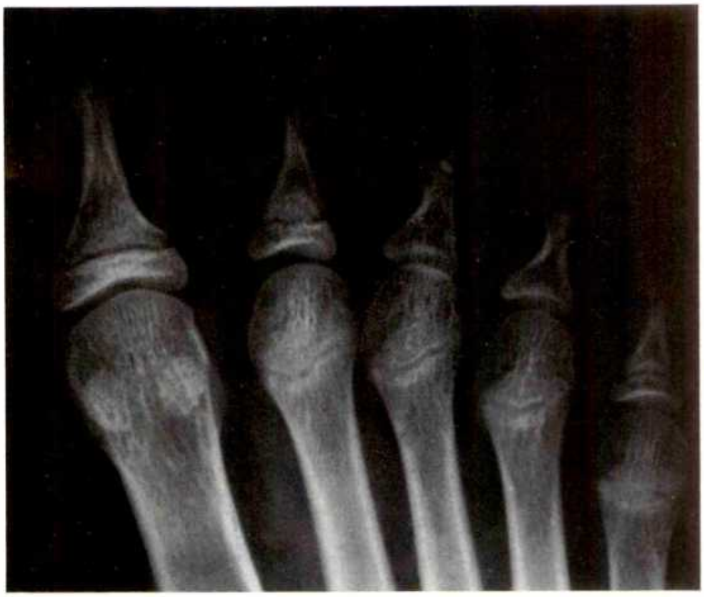

FIGURE 3. Radiograph of foot showing lack of distal and middle phalanges (Case 1).

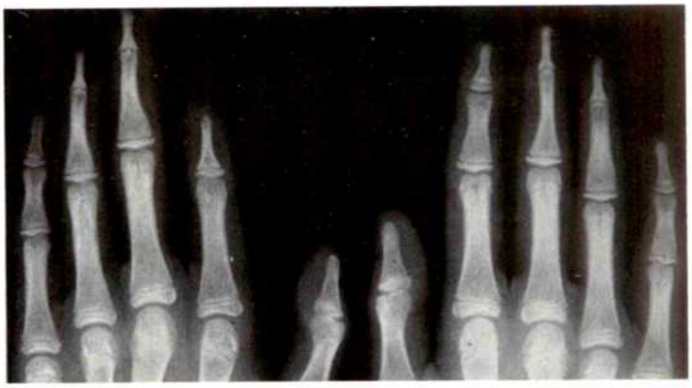

FIGURE 4. Radiograph showing slender proximal phalanges and absence of the middle phalanx on left digit II (Case 1).

## DISCUSSION

These two cases illustrate that in patients with congenital anonychia or hypoplasia, an underlying bone abnormality is often found. Kelikian (1974) states that 'one cannot conceive of a normal nail above an anomalous ungual phalanx. The nails are absent in transverse terminal defects of the digits. This is confirmed when one reviews the cases published on anonychia where X-rays have been taken. We can also reasonably conclude that there will be no nail if the

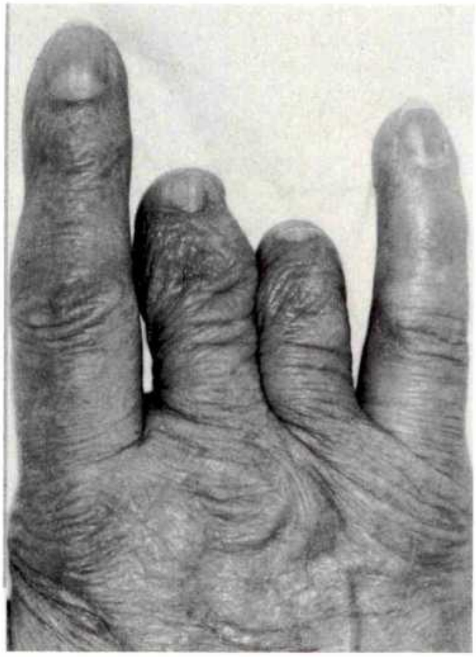

FIGURE 5. Brachydactylia of right digits III and IV with hypoplastic nails (Case 2).

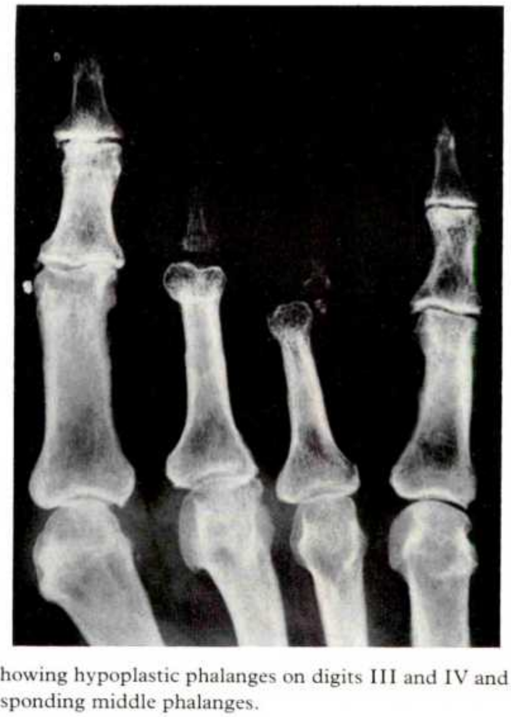

FIGURE 6. Radiograph showing hypoplastic phalanges on digits III and IV and absence and hypoplasia respectively of the corresponding middle phalanges.

distal phalanx is lacking. When the distal phalanges are hypoplastic the nails may be normal, hypoplastic or absent. In addition, the middle phalanx appears to be of importance for the formation of the nails; where it was absent on the left index finger in Case 1 there was anonychia. In Case 2 the nail was smaller where the 2nd phalanx was completely absent. In both cases we were not able to distinguish a difference in the hypoplasia of the distal phalanges.

It would appear that the development of a normal nail is dependent on the underlying bone; this dependence may extend to the middle phalanx. Anonychia or hyponychia may result when the underlying bone is either hypoplastic or completely absent. This is analogous to deformities, such as macrodactylia, which are 'nerve territory' oriented (Kelikian, 1974). In congenital onychodysplasia of the index fingers (COIF or Iso-Kikuchi syndrome) the bone is affected, but, interestingly, only one of the fingers may present the typical abnormalities (Kinoshita & Nagano, 1976; Muraoka, Tani & Miyamoto, 1977).

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# The development of lichen sclerosus et atrophicus in monozygotic twin girls

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## SUMMARY

The development of vulval lichen sclerosus et atrophicus in monozygotic twins is described. This is the first report of the occurrence of lichen sclerosus et atrophicus in two genetically identical individuals, and is considered to provide further evidence that inherited factors are of relevance in the aetiology of this disorder.

The aetiology of lichen sclerosus et atrophicus is probably mulifactorial, and appears to include a genetic predisposition in some patients, as evidenced by occasional reports in the literature of the disease affecting two or more first degree relatives in one family (Hunt, 1948; Barker & Gross, 1962; Wallace, 1971; Hofs, 1978; Murphy, Lipa & Haberman, 1982; Friedrich & MacLaren, 1984). The familial instances of lichen sclerosus et atrophicus reported to date have involved genetically similar but not genetically identical individuals (e.g. mother and child or two or more siblings). This report documents the development of lichen sclerosus et atrophicus in monozygotic twin girls, and thereby provides important additional evidence indicating that heredity may be a significant factor in the aetiology of lichen sclerosus et atrophicus.

## CASE REPORTS

MW and JW were are clinically identical twin girls aged 6 years. At the age of 4 years, MW developed sore, white areas on her vulval and perianal skin, within which haemorrhagic bullae developed; the clinical features were typical of lichen sclerosus et atrophicus (Fig. 1) and a biopsy from the vulva confirmed the clinical diagnosis (Fig. 2). At the age of 5 years, JW developed similar, although less severe, changes typical of lichen sclerosus et atrophicus affecting her vulva, but sparing the perianal skin. Neither child had any involvement of non-genital skin.

Neither the twins' younger sister, nor their parents had any evidence of lichen sclerosus et atrophicus. Neither of the twins, and none of their first degree relatives had any history, or physical or laboratory evidence of an autoimmune-related disease; one paternal cousin had

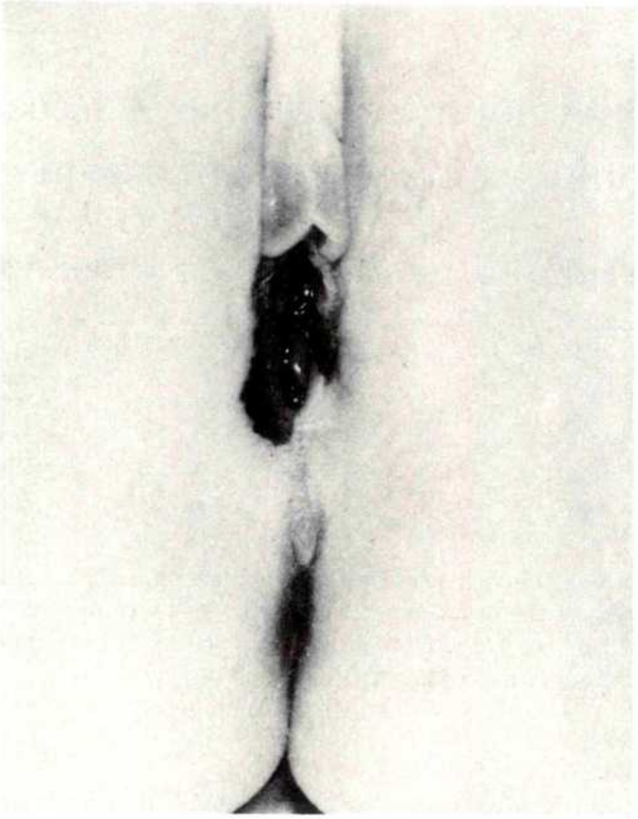

FIGURE 1. Lichen sclerosus et atrophicus of the vulva with associated haemorrhagic blister formation.

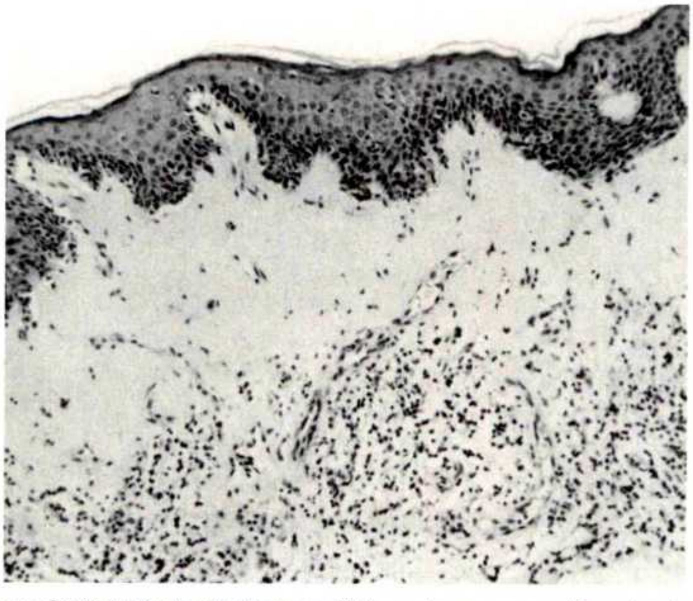

FIGURE 2. Biopsy of vulval skin showing features of lichen sclerosus et atrophicus: basal cell liquefaction degeneration, hyalinization of the upper dermal collagen and a mild inflammatory cell infiltrate in the lower dermis.

insulin dependent diabetes mellitus. No autoantibodies were detected in serum from either of the twins or from their parents. HLA-A,B and C tissue typing confirmed that the twins were genetically identical, both having the tissue types A2/3, B7/60, C3/7.

## DISCUSSION

There are a total of 15 previous instances of familial lichen sclerosus et atrophicus documented in the literature. Hunt (1948) reported three examples of lichen sclerosus et atrophicus affecting mother and daughter, Barker and Gross (1962) encountered the disease once in a mother and daughter and once in two sisters, and Wallace (1971) recorded two instances of the disease in mother and daughter and also observed the occurrence of the disease in a mother and her son, in three sisters, in a brother and sister, and in two brothers. Hofs (1978) encountered a family where the disease affected father, mother and daughter; Murphy, Lipa and Haberman (1982) described the disease in three sisters, and Friedrich and MacLaren (1984) recently reported two families in both of which the mother and two daughters were affected.

Should there be environmental factors which account for the development of lichen sclerosus et atrophicus, then it would be anticipated that any such influences would be exerted on both twins (as the twins were brought up together). It is arguable, therefore, that the development of lichen sclerosus et atrophicus in identical twins is a reflection of exposure to the same environmental factor(s) rather than a manifestation of a genetic predisposition common to both twins. However, there is as yet no known environmental cause of lichen sclerosus et atrophicus and, furthermore, the infrequency of familial instances of the disease in non-identical siblings militates against an environmental cause. In the family reported, the disease only arose in the genetically identical twins and not in the younger female sibling who would have been at the same risk of encountering any significant environmental influence. It is important, however, that the twins' younger sister should continue to be observed, as it is possible that she may develop lichen sclerosus et atrophicus at a later date.

That the familial occurrence of the disease is uncommon is evidenced both by the scarcity of familial reports in the literature, and by the observation that in a survey of 300 women and 30 men with lichen sclerosus et atrophicus, there were only two other patients in whom there was a family history of the disease in a first degree relative (Meyrick Thomas & Ridley, unpublished). Nonetheless, we consider that those familial cases which have been reported do point to a genetic factor in the aetiology of lichen sclerosus et atrophicus, and by reporting the development of the disease in monozygotic twins within 6 months of one another, we believe the case for there being a genetic aspect relevant to the aetiology of lichen sclerosus et atrophicus is strengthened.

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# Collagen and elastin changes in D-penicillamine-induced pseudoxanthoma elasticum-like skin

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## SUMMARY

A patient with cystinuria who was treated with large doses of D-penicillamine for 19 years developed skin abnormalities resembling those seen in pseudoxanthoma elasticum. Biochemical and histological examination of the dermis showed that the collagen content, the ratio of the major genetic forms of collagen and the distribution of collagen types was normal. Light microscopy demonstrated the presence of vastly increased amounts of elastin in the dermis, and the individual elastin fibres were shown by electron microscopy to be abnormal; chemical analysis showed the elastin to be poorly cross-linked. Some of the collagen also appeared structurally abnormal, and biochemically resembled that seen in the dermis of a young child with respect to cross-linking and hexosyl-lysine content. The therapy led to an increased deposition of collagen and elastin fibres which appeared abnormal, and resulted in an increase in total skin surface area. These data indicate that D-penicillamine was not fully effective in inhibiting collagen and elastin cross-linking, and appeared to prevent or inhibit the natural maturation process of the collagen.

Treatment of cystinuria with high doses of D-penicillamine has been reported to lead to abnormal elastin production in the skin (Hashimoto, McEvoy & Belcher, 1981), as well as haemorrhagic blisters (Katz, 1967), elastosis perforans serpiginosa (Guilaine, Benhamon & Molas, 1972) and cutis hyperelastica (Charlebois, Cadotte & Barbeau, 1972). The drug is

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effective in the treatment of cystinuria as it lowers urinary cystine excretion; penicillamine-cysteine disulphide is excreted in the urine instead of cystine, and as the disulphide is more soluble, urinary calculus formation is inhibited.

It is well recognized that D-penicillamine combines with the cross-link precursors of the major connective tissue proteins collagen and elastin; the drug interacts with the lysine aldehyde cross-link precursors to form relatively stable thiazolidine derivatives which preclude the possibility of intermolecular bonding of these proteins, thereby destabilizing the connective tissue matrix (Nimni, 1974).

Based on studies of the skin from a patient who had received D-penicillamine in large doses (3 g/day) for 19 years as therapy for cystinuria, this report presents data which further extend our knowledge and understanding of the action of D-penicillamine on the production of elastin and collagen in the skin.

## METHODS

Abnormal skin was obtained from the patient (male, aged 59 years) and normal skin was obtained for healthy controls (2 males, aged 63 years and a female aged 65 years) by biopsy. Samples were examined by routine light microscopy and also sectioned for routine transmission electron microscopy using standard methods.

The collagen content of the skin samples was estimated on 6 mm circular punches of known weight, by standard hydroxyproline assay (Etherington & Sims, 1981). All material other than elastin was removed from the samples by CNBr digestion in 70% (v/v) formic acid at 30°C as previously described (Light, 1982). Residual elastin was then estimated by weighing after freeze drying.

The relative content of collagen types I and III was determined by the estimation of CNBr peptides of the two types after SDS-polyacrylamide gel electrophoresis (Light, 1982). The content of the type I collagen cross-linking peptide $\alpha$1CB6 was also determined by this method (Light, 1979).

Collagen and elastin cross-links were quantified after acid hydrolysis of $^3$H-KBH$_4$-reduced skin samples (Robins, Shimokomaki & Bailey, 1973) on ion-exchange columns or high performance liquid chromatograms as previously described (Light & Bailey, 1982; Smolenski, Avery & Light, 1983).

Free and total urinary hydroxyproline were estimated on aliquots taken from a 24 h urine collection, before and after acid hydrolysis, using the standard method (Etherington & Sims, 1981).

## RESULTS

### *Clinical*

The initial clinical observation made on this patient was of an excess production and elastotic change of the skin, there being large, loose, redundant folds of skin on the chest, abdomen and back, yellowish elastotic papules on the face and neck, and a 'plucked-chicken skin' appearance of the skin in the axillae; a detailed clinical description of the cutaneous features of the patient has been reported elsewhere (Meyrick Thomas *et al.*, 1984).

### *Microscopy*

Figure 1 shows the results of routine transmission electron microscopy of the collagen and

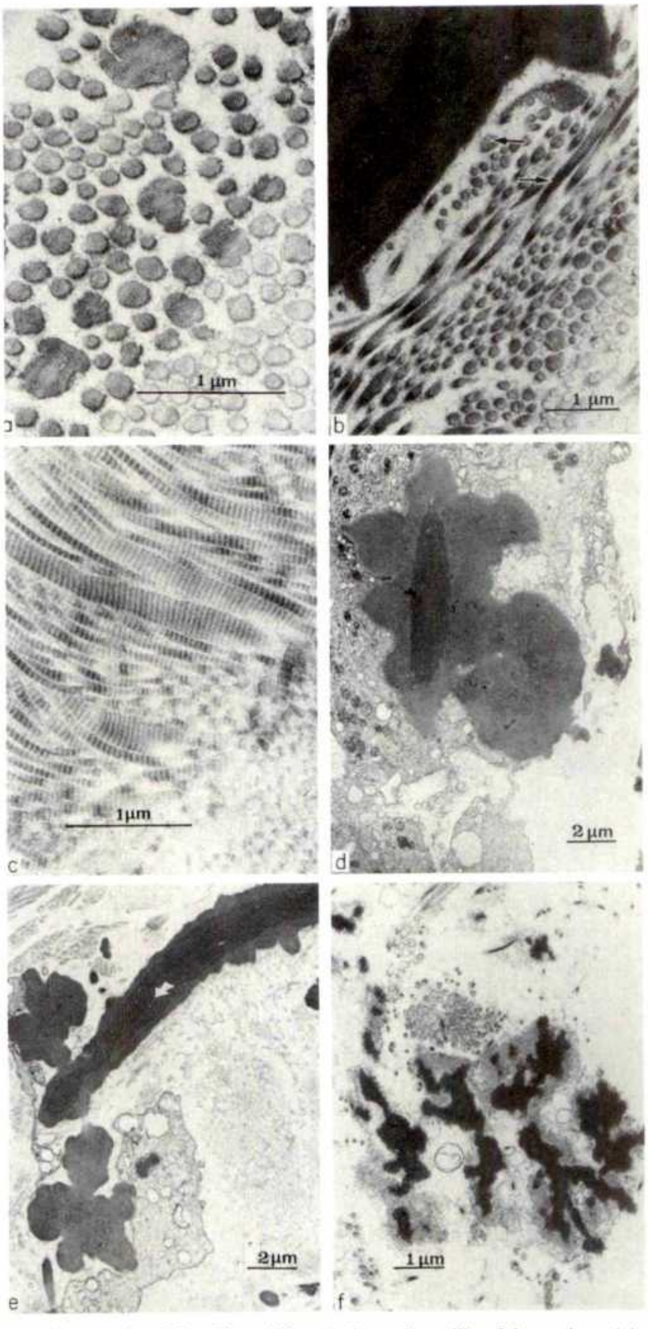

FIGURE 1. Electron micrographs of the affected dermis from the axilla of the patient: (a) transverse section of a collagen fibre in the deep dermis showing irregular, large fibrils; (b) section from mid-dermis showing 'frayed' and 'twisted' collagen fibrils (arrowed); (c) longitudinal section of dermal collagen showing variations in the size of fibrils and their irregular packing; (d) elastin fibre in mid-dermis showing irregular outline; (e) elastin fibre in mid-dermis with central, vacuolated microfibrillar content (arrowed); (f) irregular elastin fibres from deep dermis, possibly degenerate, with peripheral concentrations of microfibrils.

elastic fibres from the patient. Fibre diameters were 100–140 nm compared with those seen in normal subjects which were 70–80 nm; the variation in fibre diameter was also greater in the patient than in the controls. A proportion of collagen fibres in the affected skin had a bizarre appearance, being large and irregular in outline (Fig. 1a); some also appeared ‘frayed’ or ‘twisted’ (Fig. 1b). Packing of fibres seemed irregular (Fig. 1c) and fine fibrils were noted between ill-packed fibres (Fig. 1a). Elastin in the patient’s skin was very prominent and bizarre. The fibres were irregular in outline (Fig. 1d) compared with those seen in normal subjects, and dermal cells were often found to be in close apposition to these fibres. In many elastin fibres the microfibrillar component was seen to be concentrated in the centre of the fibre (Fig. 1d) and was sometimes vacuolated (Fig. 1e); many fibres also showed peripheral concentrations of microfibrils (Fig. 1f).

TABLE 1. Elastin and collagen content and cross-linking in pseudoxanthoma elasticum-like dermis and in dermis from normal controls. (All results are the mean of duplicate estimations)

| | | Control dermis | | |
|---|---|---|---|---|
| | | Subject | | |
| | Affected dermis | Male | Male | Female |
| Elastin content (mg/mm$^2$) | 0·47 | 0·18 | 0·14 | 0·13 |
| Desmosine (cpm/mg) | 1067 | 7880 | 6832 | nd |
| Collagen content (mg/mm$^2$) | 0·21 | 0·23 | 0·19 | 0·23 |
| Type III collagen (% of total type I and III) | 19·3 | 21·0 | 16·5 | 21·3 |
| Hydroxylysinonorleucine cross-link in collagen (cpm/mg) | 4900 | 1100 | 1290 | nd |
| Hexosyl-lysine content (cpm/mg) | 710 | 3900 | 3730 | nd |
| Cross-link precursor, 6-hydroxynorleucine (cpm/mg) | 1300 | 620 | 790 | nd |

nd: estimation not done.

*Biochemistry*

Table 1 summarizes the results obtained from the biochemical analyses carried out on dermis from the patient and from the normal controls.

The content of the elastin cross-link desmosine per unit dry weight of the patient’s skin was approximately half that of the normal control samples. This represented approximately one fifth to one eighth of the normal amount of this cross-link per mg of elastin, due to the unusually high content of this protein in the patient’s skin. The method used in the present study has been used in the past to quantify elastin in intractable tissues; however, some degree of inaccuracy must be expected due to the small size of the samples used and the limited extent of extraction. In consequence, these results are considered to indicate a massive increase in elastin content rather than as being taken as conclusive proof. This conclusion is supported by the electron microscopic findings reported above.

The collagen content of the affected dermis (Table 1) and the ratio of the two major genetic forms of collagen, types I and III (Fig. 2 and Table 1) were normal, as was the total daily urinary hydroxyproline excretion (40·8 mg/24 h—normal range 17–59 mg/24 h, Le Roy, 1967). The level of the collagen and elastin cross-link precursor, 6-hydroxynorleucine, was 2·1 times higher in the patient's skin compared with the controls, but the level of the collagen cross-link, hydroxylysinonorleucine, was more than 4 times higher in the affected skin compared with the normal controls. Conversely, in the patient's skin, the hexosyl-lysine content—which is a good marker of ageing and maturation (Kent, Light & Bailey, 1985)—was one fifth to one sixth that of the normal controls.

Related to these results was the finding that the extent of complex cross-linking by polyvalent inter-molecular bonds in the collagen was substantially lower (by approximately 3·5 fold) in the patient's skin than in the skin of the normal controls (Fig. 2). This was assessed by quantifying the amount of α 1CB6 peptide present in the affected skin from gel electrophoresis of CNBr digest of skin, as shown in (a) on Figure 2. As seen in the analysis of normal skin (b) on Figure 2, this peptide is not present in adult type I collagen due to its participation in the formation of a

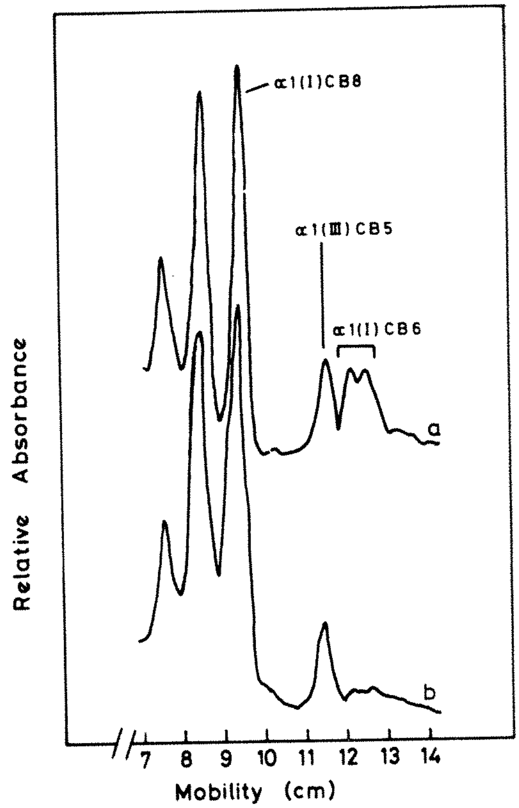

FIGURE 2. Densitometric scans of CNBr peptides of skin collagen from the dermis of the patient (a) and a normal control (b) after separation by SDS-polyacrylamide gel electrophoresis. Peptides used for quantification of types I and III collagen (α1 (I)CB8 for type I and α1(III)CB5 for type III) are shown and the cross-linking peptide α(I)CB6 is also indicated.

polymeric peptide linked by covalent cross-links, which migrates to the top of the gel (Light & Bailey, 1979).

## DISCUSSION

Previous research using both animal models and human case studies has shown that D-penicillamine has several sites of action in relation to its effect on the two major connective tissue proteins elastin and collagen. Nimni *et al.* (1974) demonstrated that animals fed the drug develop abnormalities of the skin and other soft tissues but remain unaffected with respect to their skeletons and other hard tissues. The collagen from the skin of such animals has a high aldehyde content and very poor cross-linking through acid-labile aldimine N.6-dehydro-5-hydroxylylysinonorleucine. Their bone and tendons contain a higher ratio of the acid-stable cross-link, hydroxylysino-5-keto-norleucine, a finding which has also been demonstrated *in vitro* (Siegel, 1977). At concentrations between $10^{-2}$ mol/l and $10^{-3}$ mol/l the drug inhibits lysyl oxidase, the enzyme which produces cross-link precursors (Siegel, 1977), cleaves Schiff's-bases and inhibits fibril formation.

Taken together, these data amply demonstrate how both collagen and elastin cross-linking, and therefore connective tissue structure, can be perturbed by the drug; thus it is not surprising that several clinical abnormalities of the skin have been reported in patients receiving the drug in large doses, as we have mentioned. The patient reported here developed cutaneous abnormalities which were bizarre and which have not been described previously in patients receiving high doses of D-penicillamine (Meyrick Thomas *et al.*, 1984); the cutaneous features indicated an excess production of skin which in turn suggested that D-penicillamine was causing a profound disturbance of the normal mechanisms controlling either synthesis or degradation, or both, of the connective tissue components of the skin. Study of the patient's skin thereby provided an opportunity to extend the understanding of the effect of D-penicillamine on collagen and elastin production in the skin.

We have shown that the elastin from the patient's skin is poorly cross-linked, a factor which almost certainly accounts for the abnormal structure of the fibres demonstrated on electronmicrographs from our patient's skin, and described previously by others in studies of other D-penicillamine induced dermatoses as 'bramble-bush' or 'lumpy-bumpy' elastic fibres (Kirsch & Huckill, 1977; Bardach, Gebhart & Niebauer, 1979). Electronmicroscopy also demonstrated abnormalities of the collagen fibres in the patient's dermis; the irregular and large fibres which we observed, and which have also been observed by others in other D-penicillamine-induced dermatoses (Hashimoto *et al.*, 1981) were not due to abnormal collagen content, however, nor to changes in the ratio of the major forms (types I and III) as both these parameters were found to be normal. However, it should be noted that fibrils of this type are not specific and may be found in many connective tissue diseases, such as Ehlers-Danlos syndrome and lichen sclerosus et atrophicus, and may occasionally be found in normal subjects, especially in non-cutaneous connective tissue.

The patient's dermal collagen was abnormally cross-linked compared with age and sex matched controls. However, the results did not fit the classical picture of D-penicillamine treatment. The collagen contained a 5-fold excess of the preliminary skin cross-link (the aldimine, dehydro-hydroxylysinonorleucine), normally found at high levels only in early post-natal dermis. Also elevated was the amount of cross-link precursor. This indicates that a proportion of cross-link precursor aldehydes were blocked by the drug but a significantly high proportion were not affected and were able to form an abnormally high quantity of aldimine cross-links. The skin of a young child contains 10 times the quantity of these cross-links in comparison with that of a mature adult (Robins *et al.*, 1973); the patient's level of 5 times the normal adult quantity indicates that, if newly synthesized, this collagen (like the elastin) is poorly cross-linked.

The evidence from Figure 2 indicates that the collagen in the patient's skin was not mature. The C-terminal CNBr peptide of the $\alpha 1$ chain of the type I collagen, $\alpha 1$CB6, is normally involved in the formation of the polymeric structures which stabilize and characterize 'mature' collagen (Light & Bailey, 1979). The peptide can only be seen as its monomeric form on analytical gels in samples from young patients (Robins *et al.*, 1973; Light & Bailey, 1979), or in adults in collagens with a high turnover rate, e.g. the periodontal ligament (Light, unpublished data). The high proportions of this peptide in the patient's collagen and the excessive quantity of skin confirm that the collagen is being both overproduced and is not maturing at the normal rate, if at all.

The levels of hydroxyproline in the patient's urine were normal, therby indicating a normal turnover rate for this excess skin collagen; the very low level of hexosyl-lysine residues in the affected collagen indicates a fast turnover, however. These apparently conflicting results suggest that D-penicillamine might be inhibiting both normal collagen maturation and random glycosylation so that the collagen resides in an 'immature' form in the dermis for the normal duration; this may be due to the direct binding of D-penicillamine to the Schiff's base cross-link, thereby inhibiting its further reaction to form complex 'mature' cross-links. The mechanism of inhibition of hexosyl-lysine formation remains less clear, however.

On the basis of our results we can summarize the effects of the drug in this patient. Initially, at the high concentrations after ingestion, D-penicillamine probably blocks newly formed aldehyde cross-link precursors in both collagen and elastin. At this level it may even inhibit lysyl oxidase directly—however, this is less likely, as concentrations in excess of $10^{-3}$ mol/l are necessary to block the enzyme activity efficiently (Siegel, 1977), and such high local concentrations in the dermis would not be expected. As the local concentration of the drug falls following ingestion, to a certain unknown point, no further blocking of aldehydes takes place and the collagen and elastin can cross-link normally. In both cases the cross-link concentration is evidently sufficiently low for the fibre morphology to be disrupted.

Furthermore, our results indicate that the drug inhibits the normal ageing and maturation of dermal collagen and also the random glycosylation of collagen during maturation, such that the collagen which is cross-linked, albeit at a lower level, forms abnormal fibres and does not mature normally. These biochemical findings are new, and indicate positions in the normal process of dermal collagen synthesis, secretion and stabilization other than those already recognized, at which D-penicillamine treatment can lead to abnormalities.

Since these studies were carried out, similar pseudoxanthoma elasticum-like skin changes have been reported in two other patients undergoing long term, high dose D-penicillamine therapy (Bentley-Phillips, 1985; Meyrick Thomas & Kirby, 1985), indicating that the present case is by no means an isolated one. It is likely that it is the chronic nature of the treatment and the resulting prolonged high levels of interstitial D-penicillamine in these patients that are the crucial factors causing the unusual clinical and biochemical dermal changes observed.

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# Atypical malignant melanoma of the nail apparatus

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## SUMMARY

Malignant melanoma of the nail is a serious disease with a five year survival rate of between 21% (Booher & Pack, 1957) and 50% (Graham, 1973). The diagnosis of atypical cases ultimately depends on interpreting histological material. We report here a case in which two initial biopsies of the affected nail failed to show malignant melanoma. The diagnosis was eventually made by formal longitudinal nail biopsy including matrix tissue proximal to the dystrophic area, and examination of sections cut at several levels.

## CASE REPORT

A 40-year-old Caucasian man presented with a longitudinal pigmented streak in the nail of his right thumb which had been present for two years. A biopsy through the nail plate and nail bed, including the melanonychia, did not show evidence of melanoma or any other cause of longitudinal melanonychia. The following year he suffered minor trauma to the same nail, the plate subsequently becoming distorted and lifted from the nail bed proximally; clinically there was no gross pigmentary change. A further biopsy was performed after removing the plate; again, there was no histological evidence of melanoma. The nail regrew but was grossly dystrophic (Fig. 1). At no stage was the periungual tissue affected—Hutchinson's sign was not present (Haneke, Baran & Bureau, 1984).

He was referred to Oxford and a formal 3 mm wide longitudinal nail biopsy was performed (Zaias, 1980), extending from the distal skin crease overlying the terminal interphalangeal joint to a point 3 mm beyond the free edge of the nail; at its deepest level, the biopsy contained tissue immediately superficial to bone. After the diagnosis of malignant melanoma had been substantiated histologically he underwent an amputation at the metacarpo-phalangeal joint.

### *Histology*

The entire nail biopsy was fixed in 10% formol saline and then softened for 24 h in 5% aqueous phenol before processing in paraffin wax (Fig. 2a). Many sections from different levels were cut and these were stained with haematoxylin and eosin, by the Massons-Fontana method for melanin and the periodic-acid Schiff method to identify fungi.

At the nail fold, along the nail bed and at the finger tip in some sections there were occasional foci of proliferating, pleomorphic mainly cuboidal melanocytes. The cytoplasm of these cells was clear and the nuclei had conspicuous nucleoli within their large nuclei. Many cells included

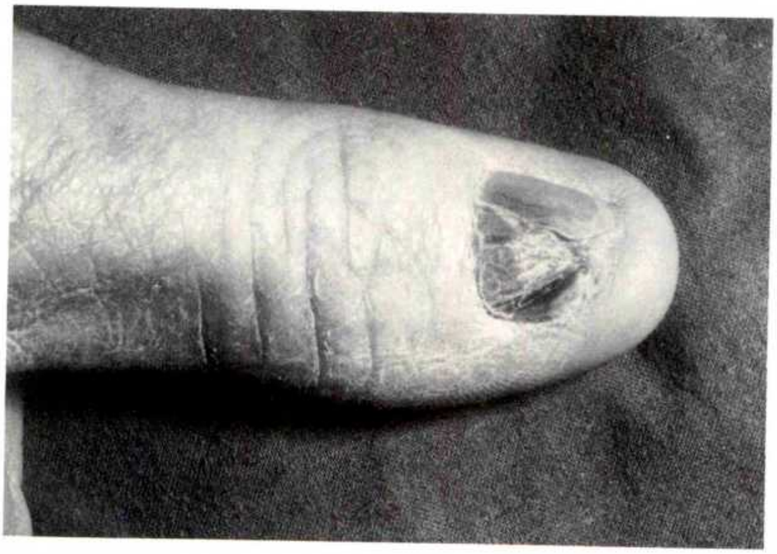

FIGURE 1. Dystrophic thumb three years after the development of a longitudinal pigmented band.

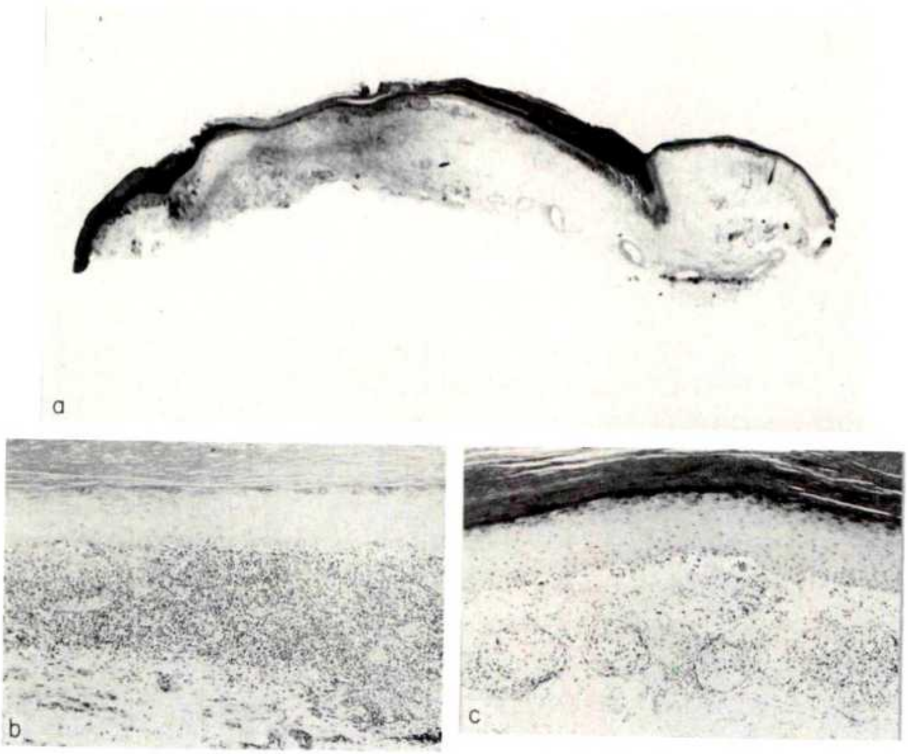

FIGURE 2. (a) Longitudinal nail biopsy. At the nail fold (right) there is some separation at the dermo-epidermal junction and a cellular infiltrate (H & E) (Original × 6); (b) no tumour is visible at this site and the appearances would be compatible with lichen planus (H & E) (Original × 70); (c) nodules of tumour lie in the dermis in the mid portion of the nail bed (H & E) (Original × 70).

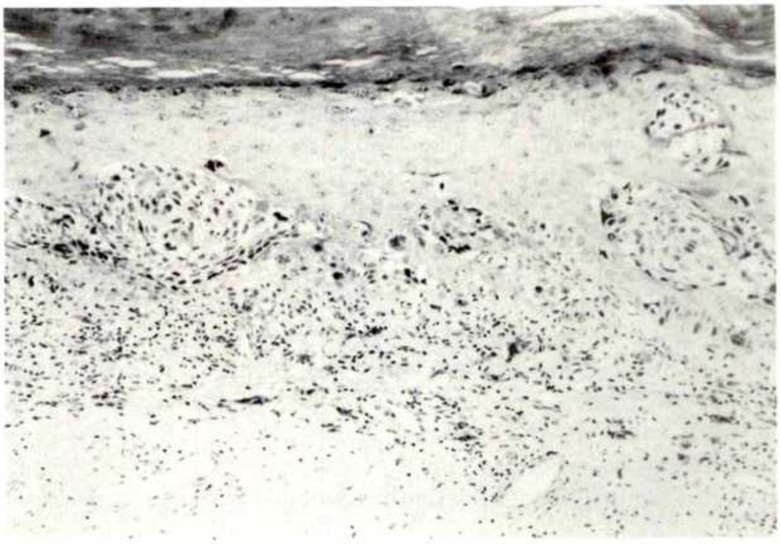

FIGURE 3. Single and collections of pleomorphic melanocytes are infiltrating the epidermis. Cluster formation is present at the dermo-epidermal junction. (H & E) (Original × 100).

a fine dusting of melanin in their cytoplasm which was most evident in the Fontana preparations. Cluster formation of the tumour cells at the dermo-epidermal junction was widespread but especially so in the region of the nail bed (Fig. 2c). Islands of similar cells were also evident in the dermis and in the nail fold, within the epidermis (Fig. 3). Along parts of the nail bed in some levels there was destruction of the basal regions of the epidermis with separation from the underlying dermis. Associated with these features as well as with some areas of obvious melanocytic proliferation there were substantial numbers of mononuclear cells, including melanophages in the upper dermis. These cells were closely applied to the epidermis and, in levels without obvious tumour, mimicked lichen planus (Fig. 2b). No fungus was recognized. Similar changes were found when the amputated thumb was examined histologically.

## DISCUSSION

The presence of an acquired, longitudinal pigmented band in the nail in a Causasian should be regarded as malignant melanoma until proved otherwise. Although gross destruction or the presence of Hutchinson's sign (pigmentation spreading into periungual tissue) make the diagnosis more likely, it is always necessary to perform a substantive biopsy. Gibson *et al.* (1957) reported that over 60% of their patients had abortive surgery before the diagnosis was made. The dangers of limited biopsy are readily apparent in this case; Figure 2a shows the complete biopsy and it is easy to see how a limited biopsy could have provided tissue showing only a lichenoid band of mononuclear cells (Fig. 2b). It is also important to cut and examine several levels. The area shown in Figure 2c is split at the dermo-epidermal junction. A biopsy including epidermis only would have failed to show the tumour. Whatever the physical signs in evidence at presentation, it is important to include proximal nail matrix tissue in the biopsy as this area will typically be the site of origin of the tumour. A healthy nail bed usually does not possess melanocytes.

Hutchinson (1886) first described subungual melanoma. A more recent report (Papachristou & Portner, 1982) highlighted the variable presentation—ulceration, pigmented band or spot, nail loss, paronychia and pain; since the signs may resemble infection, ingrowing toe-nail or trauma, the correct diagnosis may not be made until the tumour is at a late stage. These authors describe 52 patients (26 males, 26 females; median age 65 years). Eighteen had lesions on the fingers and 34 on the toes; six had pre-existing moles and some remembered previous trauma. Nail apparatus melanomas made up 3·5% of all melanomas in another series (Das Gupta & Brasfield, 1965).

In Black and Oriental races the proportion of melanomas occurring under the nail may be as high as 20%. Overall, these groups do, however, have a lower incidence of melanoma. Benign naevoid, fixed pigmented bands are, however, very common, particularly in negroid individuals. In Puerto Ricans (Vazquez, Ramos & Sanchez, 1984) a study over 22 years revealed three subungual melanomas out of a total of 19 acral cases. The rare occurrence of secondary melanoma deposits in the nail bed is apparently associated with widespread metastatic spread (Retsas & Samman, 1983).

This particular case emphasizes the need to understand the normal nail apparatus anatomy to enable adequate tissue to be taken for the early diagnosis of malignant melanoma. The poor prognosis generally described for melanoma in the nail apparatus may in part be due to the many cases which get appropriate treatment only at a late stage.

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*British Journal of Dermatology* (1986) **114**, 393–397.

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## Neutrophilic urticaria

MADAM, I was very interested indeed to read the article on neutrophilic urticaria by Peters and Winkelmann (*British Journal of Dermatology* 1985, **113**, 25). The authors are to be congratulated on delineating a group of patients with chronic urticaria which shows a characteristic histology and is associated with dermographism in at least seven out of the 10 patients. The more we chip at the mass of so-called 'idiopathic' chronic urticaria, the more of these sub-sets will be determined.

For many years there have been conflicting opinions on the prevalence of dermographism in chronic urticaria. Clearly some 5%–6% will have dermographism, as this is the prevalence in the normal population. In addition, there is a clinical sub-set of chronic urticaria which starts as a sudden acute attack, associated with marked dermographism. This state may then persist for a few months and, therefore, eventually fall into the category of chronic urticaria. This is seen particularly with penicillin-induced urticaria and also with parasitic causes, for example the liver fluke. The inclusion of such cases will obviously increase the figures for the prevalence of dermographism. Of Peters and Winkelmann's 10 cases, four had had urticaria for only 2–4 months and four had histories of reaction to drugs. It would seem likely that some of these patients would fall into this clinical sub-set. It would also be very instructive to know the prevalence of dermographism in the other 231 patients mentioned by these authors, as this will affect the significance of the association with dermographism in the 10 patients.

The authors mention that three cases had pressure-induced weals, and more clinical information is needed to understand the nature of these. Two of these patients had dermographism, and, in dermographic subjects, weals and deeper swellings may occur at sites where pressure or rubbing has occurred but may not have been noticed by the patient. These are likely to last a short time, usually 0·5–2 h. Pressure induced weals in chronic urticaria can occur at the height of the eruptions and probably the pressure only determines the position of the weal (Köbner weals). True pressure urticaria comes up 4–6 h after pressure, and lasts 24 to 48 h and may or may not be associated with chronic urticaria.

It is difficult to get a good picture of the clinical state of the patients showing the particular histology described.

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R.P.WARIN

## Reply

MADAM, We appreciate Dr Robert Warin's comments on the article on neutrophilic urticaria. We recognize his many contributions to the field of urticaria and are happy to have his ideas. We share many of his questions as to how to identify these patients clinically. An additional manuscript, including clinical photographs of the patients from the collection of St. John's Hospital, will be published shortly. The clinical appearance is typical for chronic urticarial lesions, and physical urticarial changes were found in the second series of patients also.

We believe the major question is: are all the lesions in these patients neutrophilic urticarial in histology, or do they make a transition from neutrophilic to lymphocytic urticarial lesions, which would be more typical of chronic urticaria? This question can only be answered by multiple biopsies from patients with neutrophilic urticaria, and we hope that this can be accomplished in the near future.

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R.K.WINKELMANN

## Erythema nodosum associated with invasive amoebiasis and giardiasis

MADAM, Erythema nodosum is associated with a multiplicity of diseases and conditions. Common causes include sarcoidosis, streptococcal pharyngitis and sulphonamide ingestion. We report an unusual case where erythema nodosum was associated with amoebic dysentery and giardiasis.

A 29-year-old British medical practitioner attended the Hospital for Tropical Diseases, London, with painful red nodules on both legs which had been present for 24 h. She gave a three-week history of diarrhoea acquired while on holiday in South-East Asia, and 5 days prior to being seen had noticed blood mixed in with her stools. She was taking chloroquine 300 mg weekly for malaria prophylaxis, and was also on a biphasic norethisterone-ethinyloestradiol contraceptive preparation which she had been taking regularly for 6 years. There had been no sulphonamide ingestion and there was no history of sore throat. General examination was normal apart from classical erythema nodosum lesions on both legs. On sigmoidoscopy there were several discrete ulcers throughout the rectum with normal intervening rectal mucosa. Cysts and trophozoites of *Giardia lamblia* were found on direct stool microscopy. Haematophagous motile trophozoites of *Entamoeba histolytica* were identified in rectal mucosal scrapes. Amoebic serology (indirect fluorescent antibody test) was positive at a titre of 1:80. Chest X-ray was normal and stool cultures were negative. Treatment was commenced with metronidazole 800 mg three times a day for 5 days, followed by diloxanide furoate 500 mg three times a day for ten days; contraceptive medication and malarial prophylaxis were continued. Twelve days later the patient was asymptomatic, bowel action was normal and the cutaneous lesions had disappeared. Repeat stool microscopy was negative.

The occurrence of erythema nodosum with inflammatory bowel disease is well recognized, and it may also occur in intestinal infection with *Yersinia enterocolitica* (Mygind & Thulin, 1970), *Campylobacter sp.* (Ellis *et al.*, 1982), and *Salmonella typhimurium* (Morrison *et al.*, 1983). Erythema nodosum has not, to our knowledge, been described with invasive amoebiasis or giardiasis. One can only speculate in this case as to which of the two protozoa was responsible, as both respond equally to high dose metronidazole. Erythema nodosum can rarely be associated with oral contraceptive medication (Bombardieri *et al.*, 1977), but continuing challenge with this agent was not associated with recrudescence, which eliminates this potential cause. Erythema nodosum is considered to be a lesion mediated by immune mechanisms, which develops in response to a variety of antigenic stimuli. We feel that *Entamoeba histolytica* or *Giardia lamblia*, or both, are contenders for a place in the long list of agents known to be associated with this cutaneous lesion.

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### ACKNOWLEDGMENT

We thank the laboratory staff of the Hospital for Tropical Diseases for microscopical and serological investigations.

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## Lichen aureus

MADAM, In the review on lichen aureus by Price *et al.* (*British Journal of Dermatology*, 1985, **112**, 307) it was asserted that this dermatosis is limited to one or two sites. Although most previous reports have emphasized this localization, Farrington (1970) documented two cases of lichen aureas with multiple lesions on both thighs, chest and the right arm. In addition, we have encountered two patients where the characteristic lesions of lichen aureus were widespread.

*Case 1*

A 13-year-old boy first developed a pigmented patch 17 days after birth, adjacent to the left-sided scar of a bilateral inguinal hernia repair. This lesion gradually enlarged over several years and further similar patches appeared on both buttocks, the right groin and the anterior aspect of the left lower leg. All these lesions have persisted unchanged, apart from the one on the leg which has regressed over the past two years. He was otherwise asymptomatic, with no relevant drug or family history. Cutaneous examination revealed striking golden brown, non-elevated patches studded with multiple petechiae at all the aforementioned sites. In addition, papules were noted at the periphery of the lesion on the right buttock. There was considerable variation in the size of these lesions with the largest measuring 14 cm at its maximum diameter. Standard haematological and biochemical investigations were normal as was a Hess test. A skin biopsy taken from a lichenoid papule on the right buttock showed mild epidermal change with hyperkeratosis and focal parakeratosis. The principal findings, however, were in the upper dermis, which contained a band of heavy lympho-histiocytic infiltrate together with extravasated erythrocytes, and haemosiderin laden macrophages, confirmed by Prussian blue stain, beneath the lichenoid infiltrate.

*Case 2*

A 43-year-old lorry driver presented with a 10 year history of a persistent non-pruritic rash in both axillae, groins, behind the knees and on the right foot. There was no history of drug ingestion, and he was otherwise well. Examination showed the characteristic golden coloured lesions of lichen aureus at all the above sites. Investigation revealed no bleeding diathesis, and a skin biopsy showed a normal epidermis and dermal changes similar to the previous case, although the lympho-histiocytic infiltration was less intense.

Although lichen aureus is usually confined to one region of the body, these two cases clearly illustrate that this eruption can be extensive.

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REFERENCE

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## Longitudinal melanonychia during photochemotherapy

MADAM, Further to the letter concerning longitudinal melanonychia and lichen planus (Baran *et al.*, *British Journal of Dermatology* 1985, **113**, 369) we would like to report the occurrence of a case during treatment of psoriasis with photochemotherapy (PUVA).

A 38-year-old Caucasian woman with longstanding extensive plaque psoriasis commenced twice weekly PUVA treatment in a graded regimen. Her nails were not involved with psoriasis and there were no nail fold naevi. After 6 weeks she noticed development of brown lines extending from the lunula to the distal nail plate. The bands darkened progressively for the 5-month duration of PUVA (Fig. 1) then grew out

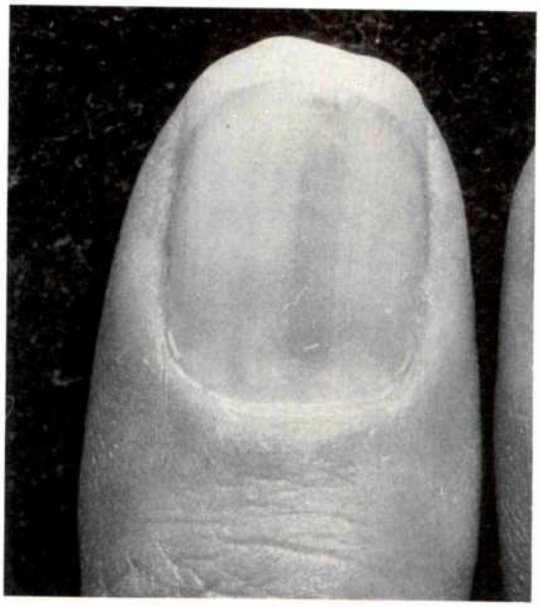

FIGURE 1. Longitudinal melanonychia of the left thumb nail.

over the following 6 months. Further bands developed during a subsequent course of PUVA, although these were not at exactly the same sites as previously. Close examination of the terminal nail plate showed retained pigment suggestive of melanin incorporation into the nail plate keratin. It is presumed that theques of nail matrix melanocytes were activated during PUVA treatment. Toe nails were unaffected and there was no family history of melanonychia.

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## Acne in a kidney transplant patient treated with cyclosporin A

Madam, Cyclosporin A (CyA) is now an accepted immunosuppressive agent used for preventing rejection episodes in human kidney transplantation. Although cutaneous side effects have been described since its introduction (Harper *et al.*, 1984), evaluation of the exact role of this drug in inducing them is difficult, due to the frequency of combined use of corticosteroids and other immunosuppressive agents in current clinical practice. We describe here a comedonal acne observed in a 35-year-old woman given a kidney graft from a cadaver donor and treated with CyA only.

The patient had renal insufficiency due to chronic glomerulonephritis, and required dialysis. She was grafted with a cadaver donor kidney at the age of 34.

The therapeutic regimen was CyA at a dose of 5 mg/kg/day intravenously for the first 3 days, followed by an oral dose of 13 mg/kg/day for 20 days then tapered by 2 mg/kg/day at the end of the first month, and every 30 days thereafter, down to a final maintenance dosage of 5 mg/kg/day from the fourth month onwards. On the day of transplanation only, she was given a single 500 mg dose of methylprednisolone intravenously. No steroids were given after this. Plasma creatinine levels became normal within 5 days after transplantation and remained stable during the entire follow-up period. Whole blood CyA levels were 1600 ng/ml during the second month (normal levels about 400 ng/ml).

The patient began to exhibit comedonal acne at the beginning of the third month. She had never applied any comedogenic cosmetics. A dermatological evaluation was made during the fourth month. At examination, the patient had acne mostly distributed over her forehead, cheeks and peroral areas. Lesions were numerous whiteheads, some blackheads, several epidermal cysts, some mildly inflamed papules and a few pustules. The lesions did not worsen during her menses. To exclude any endocrinological abnormality which might be responsible for the appearance of acne in an adult woman, we determined LH, FSH, TSH, $T_3$, $T_4$, prolactin, plasma cortisol, dehydro-epiandrosterone-sulphate, progesterone, 17-OH-progesterone, testosterone, plasma and urinary electrolytes and urinary 17-ketosteroids. These were all within normal limits. Ovarian ultrasonography did not reveal any abnormalities. Topical therapy with Tretinoin, started in the fourth month, ameliorated the acne lesions within one month.

At that time she was still taking oral CyA at a dosage of 300 mg. It is well known that CyA directly activates the follicles leading to a diffuse hypertrichosis independent of endocrinological stimuli (Pendry & Alexander, 1982; Kopsa *et al.*, 1985). In a recent review of our series of 67 cadaver kidney recipients, we observed a high incidence of follicular alterations (hypertrichosis 60%, epidermal cysts 28%, pilar keratosis 21% and acne 15%) (Bencini *et al.*, 1985). However, due to the combined use of steroids and CyA in that series of patients, it was difficult to differentiate the side effects induced by steroids from those induced by CyA. In the present case the patient did not receive any steroids as a maintenance therapy. We have suggested (Bencini *et al.*, 1985) that CyA, in addition to inducing an increase in hair growth and hair keratinization (Pendry & Alexander, 1982), could also be responsible for the induction of acne and other follicular alterations by the same mechanism, i.e. increased keratinization of the upper part of the outer epithelial layer of the follicle. Moreover, Palestine, Nussenblatt and Chan (1985), who found alterations in apocrine glands induced by CyA, suggested that it might equally affect several cutaneous structures embryologically derived from the primordial hair cells.

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## *Book Reviews*

**Illustrated Encyclopaedia of Dermatology.** L.FRY, F.T.WOJNAROWSKA and P.SHAHRAD (1985) 2nd Edition. Lancaster: MTP Press. Pp. 575. Price £35.00.

The authors, who have wide experience in clinical dermatology and investigative work, have written this book for clinicians and intend it to fill the 'gap' between current, smaller texts on dermatology and standard, large reference books. Aspiring dermatologists should perhaps be encouraged to leap across that gap as quickly as possible but this book will be useful for those requiring a bridge) and it may also be appropriate to the needs of Membership candidates and interested general practitioners.

The format of 58 chapters, with the subjects listed alphabetically, is clear and the sub-headings facilitate quick reference. The text, supplemented by 16 tables, is succinct, but contains detailed descriptions of the presentations of diseases, including multisystem disorders. There are 240 black and white photographs, but on close inspection a few are repeated in different sections and the quality of a few others is dubious. There are, additionally, 43 diagrams illustrating the surface distribution of conditions and, in general, these are clear and helpful. The value of the histograms and diagrams illustrating the age incidence and sex ratio of the conditions is less certain, since this information, in many instances, could have been given in a sentence. Perhaps it might have been preferable to have used the space for key references to literature which could amplify the statements made in the sections on aetiology and treatment.

M.I.WHITE

**Searching the Medical Literature: A Guide to Printed and Online Sources.** J.WELCH and T.A.KING (1985) London: Chapman & Hall. Pp. 154. Price £15.00.

Medicine is fortunate in the number of bibliographic reference sources available. Some, such as the Index Medicus, have been produced for many years and most physicians know how to prepare a preliminary bibliography. However, the number of medical journals has grown so fast that there are now some 10 000, and computer-aided techniques are being increasingly used by librarians and are providing methods of searching the literature faster and more comprehensibly.

Mr Welch and Mr King, who are both at the Wessex Medical Library in the University of Southampton, have provided a practical introduction to these modern methods which will be of great interest to medical librarians and to medical authors who seek their help.

Mr D.E.Hands, of the Southampton General Hospital, contributes a useful chapter on Drug Information Services.

This book can be strongly recommended and is essential reading for medical authors, many of whom may be unaware of the extent to which new information technology can save them time and increase their efficiency.

A.ROOK

**Topics in Topicals** R.MARKS (1985) Lancaster: MTP Press. Pp. 130. Price £25.00.

Books of this type—based on symposia proceedings—often lack cohesion. This book, however, does have a strong central theme, and contributors who are well-recognized experts in their fields.

Quite correctly, Professor Marks considers both cosmetics and topical pharmaceutical products together, and there are good sections on the testing of topical drugs and cosmetics for toxicity, the psychorheology of topical products, microbiological contamination of creams and skin function. The section on acne caused by topical agents is also full of useful facts and points of interest.

At £25.00 the book is expensive, but should prove a useful primer for all training dermatologists or those involved in cosmetic and pharmaceutical formulation. It provides a lot of information in a relatively condensed and well-referenced form.

J.D.WILKINSON

*British Journal of Dermatology* (1986) **114**, 399–400.

# *News and Notices*

### British Society for Dermatopathology — Call for Abstracts

The next meeting of the British Society for Dermatopathology will be held prior to the summer meeting of the British Association of Dermatologists in Sheffield. The meeting will take place on the afternoon of Tuesday, 8 July and the morning of Wednesday, 9 July, at the Octagon Centre. There will be a dinner on the evening of 8 July and inexpensive overnight accommodation is available. The theme of the meeting is melanocytic lesions, and the two guest speakers are Dr David Elder from Philadelphia and Dr Richard Sagebiel from Los Angeles.

There will be an opportunity to offer free communications of 10 minutes in length on any dermatopathological topic. Will those who wish to present such communications please send an abstract of 200 words or less to Dr Philip McKee, Department of Histopathology, St Thomas's Hospital Medical School, Lambeth Palace Road, London SE1 7EH, U.K. by 31 March 1986. Further details of the meeting can also be obtained from Dr McKee.

### Occupational Skin Disease Course 7–9 May 1986

This course aims to cover the more common occupational skin disorders, placing them in context with the working environment. Visits have been arranged to places of work which present potential dermatological problems. The course will be of particular interest to senior registrars and newly appointed consultants in dermatology.

Further information may be obtained from Dr Iain Foulds, Institute of Occupational Health, University Road West, P.O. Box 363, Birmingham B15 2TT, U.K. Telephone 021-472 1301, extension 2110.

### Dermatopathology Self-assessment Workshop

The above workshop is to be held on 11 October 1986 at the Cleveland Clinic Foundation. For further information please contact the course director: Wilma F. Bergfeld, M.D., Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44106, U.S.A. Telephone: (216) 444-5722.

### National Eczema Society Annual General Meeting

The above meeting will be held on Saturday, 26 April 1986 at 2.30 p.m. when the Keith Holden Memorial Lecture will be given by Dr T. David, Senior Lecturer, Department of Child Health, University of Manchester. For further information, please contact John Randall, Information Officer, National Eczema Society. Telephone 01-388 4097.

## The Seventh CIRD Symposium—Advances in Skin Pharmacology

The Seventh CIRD Symposium will be held in Nice from Friday, 26 September to Sunday, 28 September 1986. The topic will be: *Skin Pharmacokinetics*. This symposium will focus on skin pharmacokinetics in both animals and man for topically applied drugs. Drug kinetics in the skin after systemic application will also be considered. Emphasis will be placed on the nature of the skin barrier; *in vivo/in vitro* correlations and models and their standardization. For papers or poster presentations, please send approximately 300 word abstracts or requests for information to: Dr B. Shroot, Centre International de Recherches Dermatologiques, Sophia Antipolis, F-06565 Valbonne, France, Telephone 93957070, Telex 461 030, by 30 April 1986

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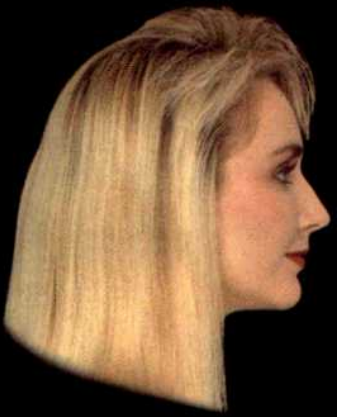

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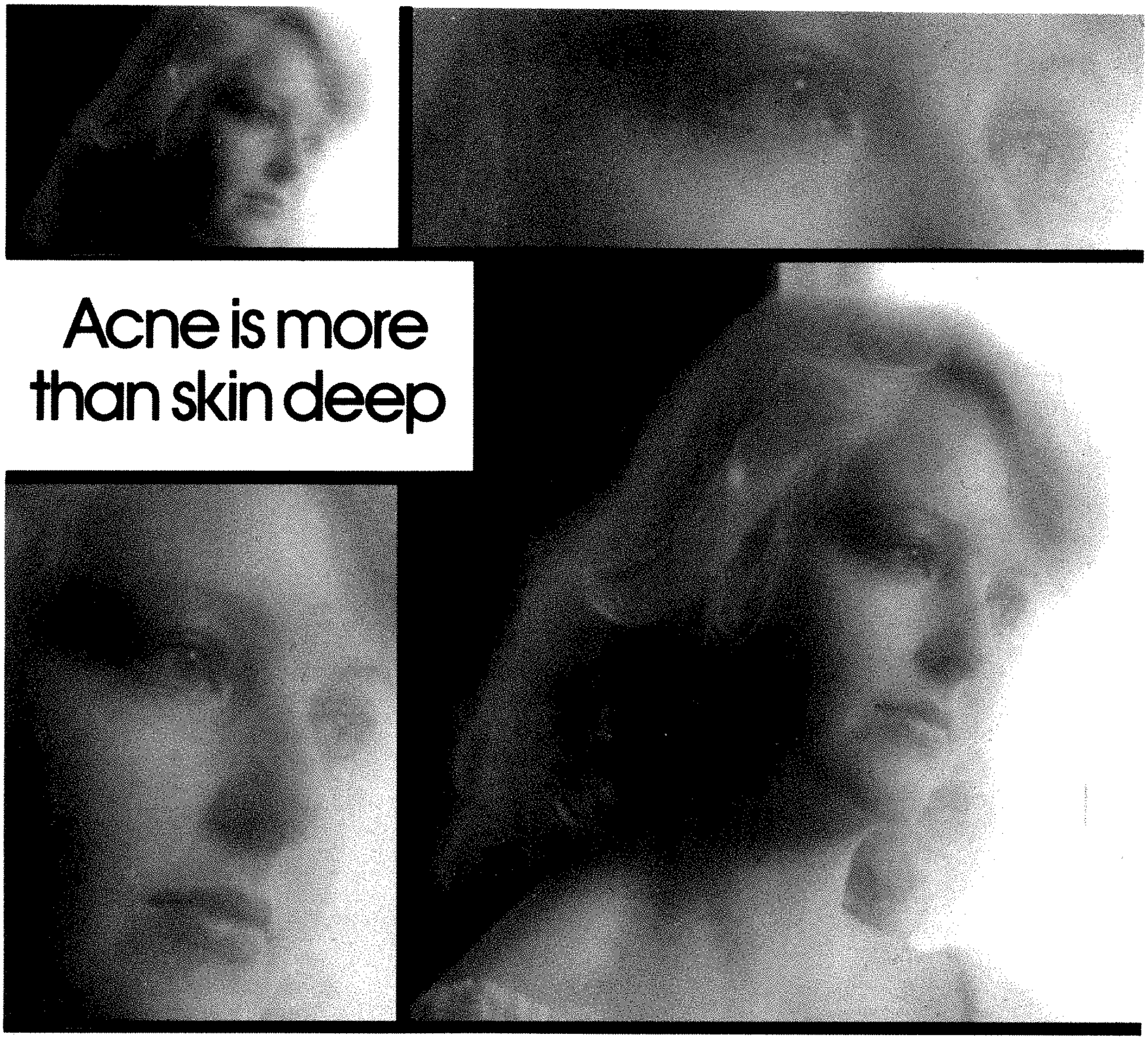

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|---|---|---|---|---|---|
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| kilogram(s) | kg | centimetre(s) | cm | millilitre(s) | ml |
| milligram(s) ($10^{-3}$g) | mg | second(s) | s | gravitational acceleration | *g* |
| microgram(s) ($10^{-6}$g) | μg | cubic millimetre(s) | $mm^3$ | micrometre(s) | μm |
| nanogram(s) ($10^{-9}$g) | ng | millimetre(s) | mm | per cent | |
| picogram(s) ($10^{-12}$g) | pg | millicurie(s) | mCi | isotopic mass number placed as | $^{14}$C |
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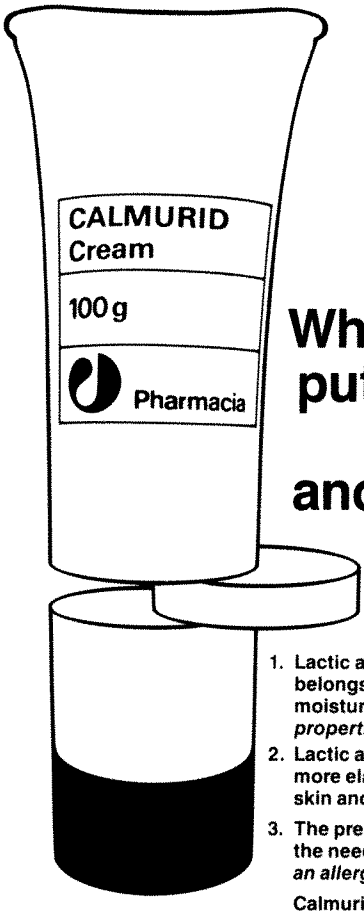
*Presentation:* An evaporative collodion paint containing 16.7% Lactic Acid BP 16.7% Salicylic Acid BP and 66.6% Flexible Collodion BP. *Indications:* For the treatment of warts, particularly plantar warts. *Directions for Use:* Paint the wart, allow to dry and cover with a plaster. Leave for 24 hours. Remove collodion by rubbing with a piece of pumice stone and repeat daily. *Contra-indications:* Not to be used on the face, anal or perineal regions. *Precautions:* Keep away from eyes. *Package Quantity:* 10ml amber glass bottle with applicator. *Basic NHS price:* £1.61. *Product licence Number:* PL 0173/5006. Salactol is a registered trade mark.



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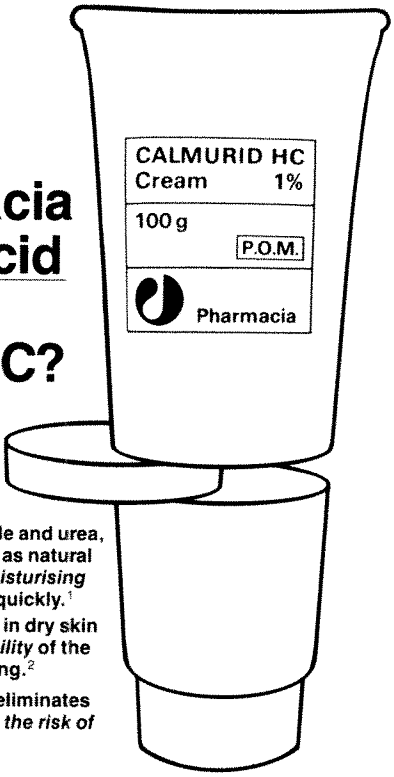

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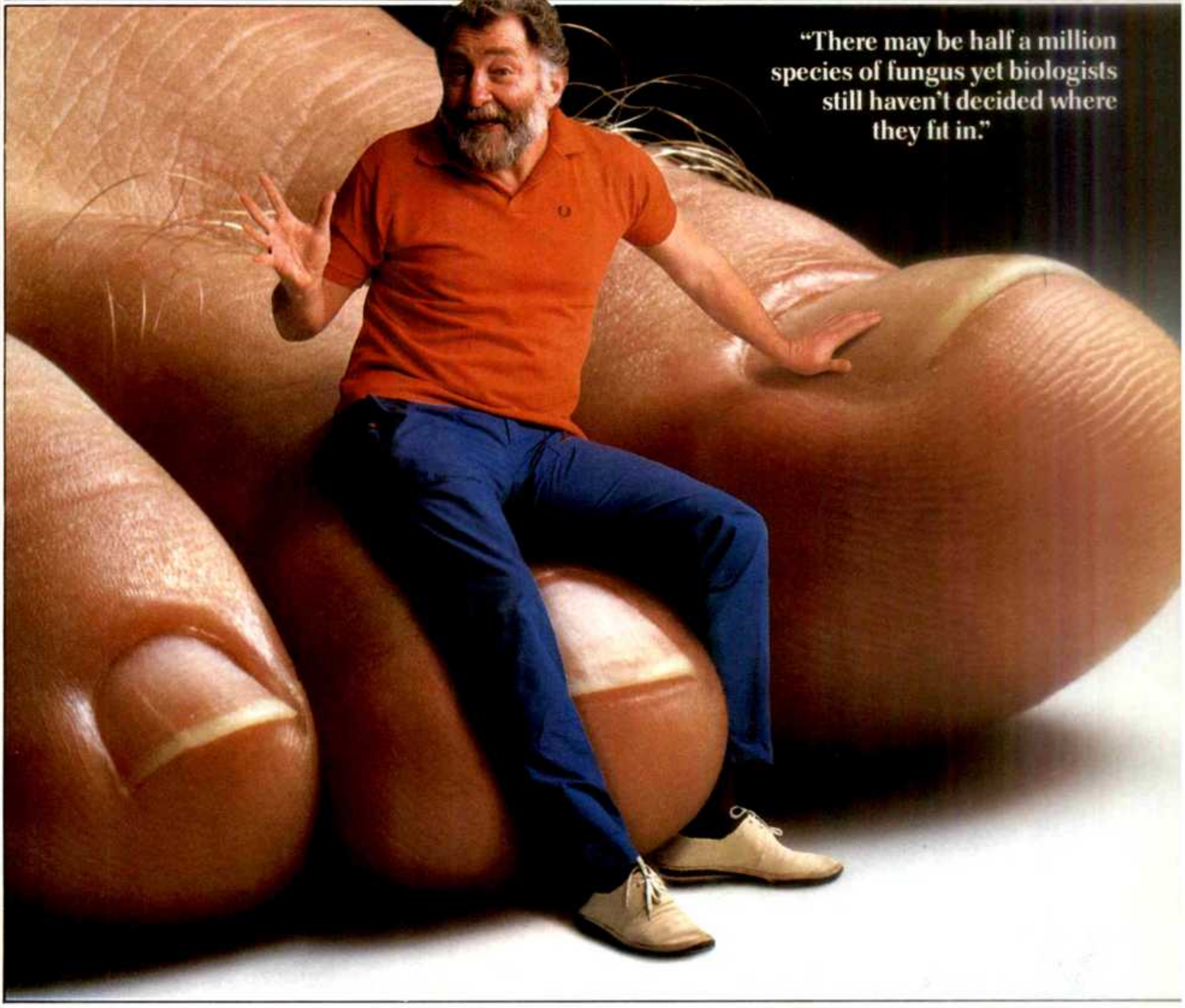
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**References:** 1 Lassus A *et al. Br J Dermatol* 1983; **108:** 195-198. 2 Tanenbaum L *et al. Arch Dermatol* 1984; **120:** 216-219. 3 Tanenbaum L *et al. Int J Dermatol* 1983; **22:** 318-320.

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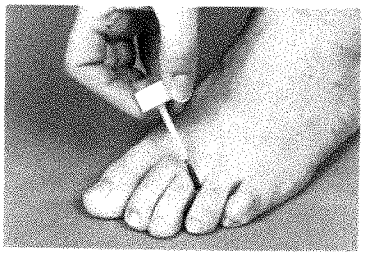

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**Legal category:** P
**Package quantities:** Bottles containing 18 ml with brush fitted in cap.
**Further information:** Nil
**Product licence number:** 0118/5030
**Basic NHS price:** £0.57 per 18 ml bottle

*Reference*

1 Krebs HA, Wiggins D, Stubbs M, Sols A, Bedoya F. Studies on the mechanism of the anti-fungal action of benzoate. Biochem J 1983; **214**: 657-63

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**Contra-indications, warnings etc** Do not apply to facial or anogenital warts. Avoid contact with the eyes. Only apply to the affected area. Keep away from naked flames.
**Pharmaceutical Precautions** Store in cool place. Highly inflammable.
**Legal Category** P
**Package Quantities** 5g Tubes
**Further Information** Warts are contagious and any person suffering from warts should always use their own towel. Most warts will disappear after 6 to 12 weeks of treatment with Cuplex gel, providing instructions are carefully and consistently followed. Where, however, the wart continues to increase in size after 6 weeks treatment and the patient has not consulted a doctor, the patient should be advised so to do.
**Product Licence Number** 0033/0100
**Basic NHS price** 5g gel £1.83

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Basispræparater til
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DK-Herlev, Marts 1986

**Theodor Nasemann scholarship for dermatovirology.**

The director of the university skin clinic Hamburg and Basoderm a/s have founded the Theodor Nasemann scholarship for dermatovirology with the purpose of intensifying research activity in the dermatovirological field.

Basoderm a/s and the director of the university skin clinic Hamburg have introduced the scholarship in order to bring recognition to important papers in the field of dermatovirology and also to afford young researchers the opportunity of learning modern methods of investigation and furthering dermatovirological research in laboratory studies.

The award, which is restricted to persons less than 40 years of age, consists of a grant of DM 20.000 and a three months research scholarship. It will be allocated to the candidate who is judged to have submitted the best paper on a dermatovirological subject.

The grant commitee invites nominations for the grant and scholarship from the directors of dermatological clinics in the different Western European countries and from research or other scientific institutions or departments in the same field. Nominations are to be submitted by 1.6.1986 to:

Prof. Dr. Med. Theodor Nasemann
Direktor der Universitäts-Hautklinik
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All documents concerning candidates will be returned to senders after a decision on allocation of the grant has been reached.

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In 1984 Dr. Med. Gunhild Lange Wantzin, Dept. of Dermatology, Bispebjerg Hospital in Copenhagen, was awarded the grant.

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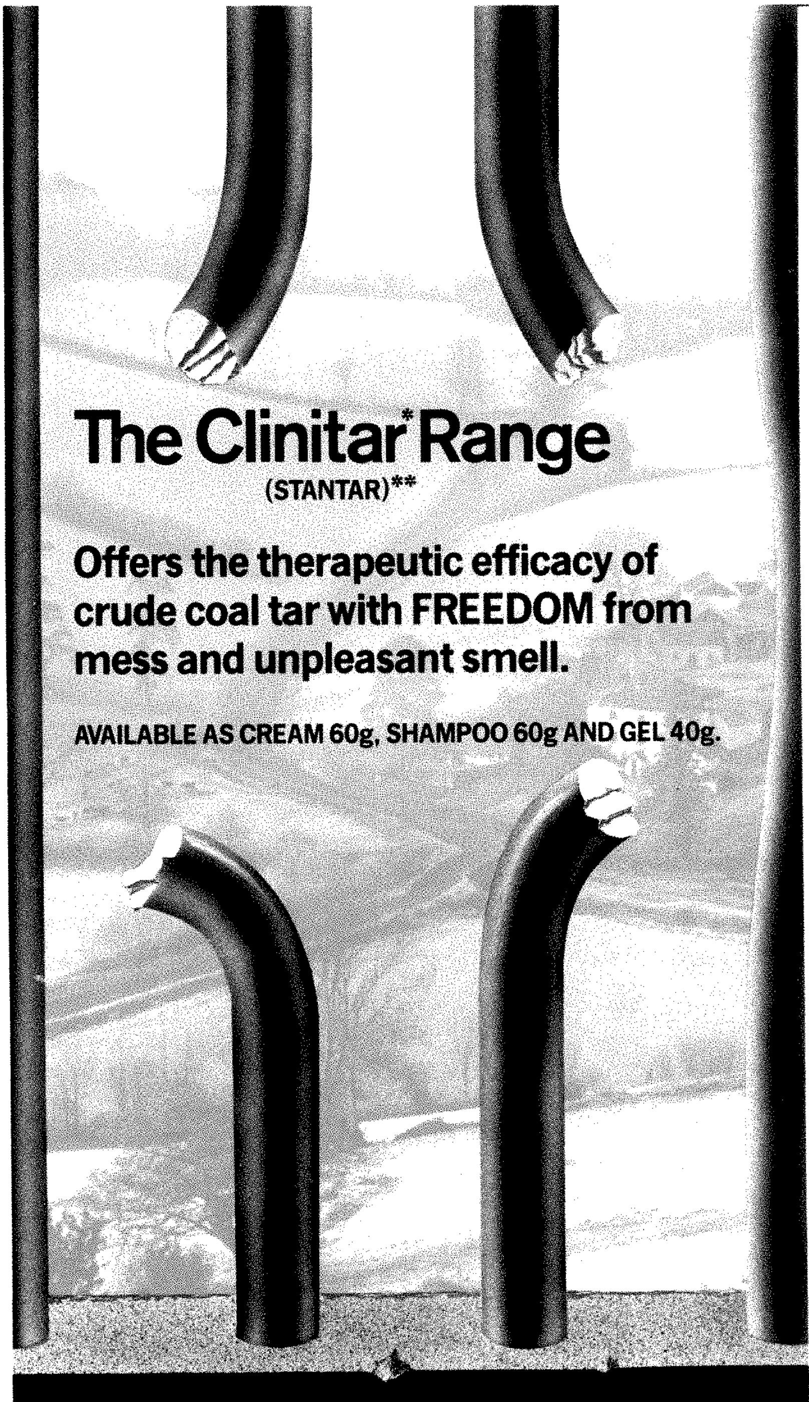
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PRESCRIBING INFORMATION

## Clinitar Cream

**PRESENTATION** Greenish-brown oil in water cream containing 1% Stantar
**USES** For the treatment of sub-acute and chronic psoriasis and eczema
**DOSAGE AND ADMINISTRATION** Apply to the affected areas once or twice daily
**CONTRA-INDICATIONS AND WARNINGS** The use of Clinitar cream is not recommended in generalised pustular psoriasis, infections of the skin, or for patients allergic to coal tar. Coal tar can sensitise the skin to U.V. light, leading to sunburn. Clinitar cream should therefore be used cautiously in the sun. Before any irradiation treatment, Clinitar cream should be removed completely
**PHARMACEUTICAL PRECAUTIONS** None
**LEGAL CATEGORY** P
**PACKAGE QUANTITIES** 60g tube
**FURTHER INFORMATION** Clinitar cream contains Stantar, which is an extract of coal tar standardised to have a photosensitising activity corresponding to that of crude coal tar. By using Stantar instead of crude coal tar a product is obtained which is easy to apply, has no unpleasant smell, and is cosmetically inconspicuous
**PRODUCT LICENCE NUMBER** 0033/0110
**BASIC NHS Cost** £2.73

## Clinitar Shampoo

**PRESENTATION** A clear brown shampoo with a slightly greenish fluorescence containing 2% Stantar
**USES** For the treatment of pityriasis simplex capitis (dandruff), seborrhoeic dermatitis of the scalp, and psoriasis of the scalp.
**DOSAGE AND ADMINISTRATION** Moisten the hair and rub a little shampoo into the hair and scalp. Rinse and repeat the treatment, this time producing a heavy lather. Leave the lather in the hair for 5 minutes after the second treatment and then rinse carefully. Use 1-3 times a week.
**CONTRA-INDICATIONS AND WARNINGS** The use of Clinitar shampoo is not recommended for patients allergic to coal tar. Avoid getting shampoo into the eyes.
**PHARMACEUTICAL PRECAUTIONS** None. Store at room temperature
**LEGAL CATEGORY** P
**PACKAGE QUANTITIES** 60g tube
**FURTHER INFORMATION** Clinitar shampoo contains Stantar, which is an extract of coal tar standardised to have a photosensitising activity corresponding to that of crude coal tar. By using Stantar instead of crude coal tar a product is obtained which is easier to rub in and wash out and has a pleasant smell
**PRODUCT LICENCE NUMBER** 0033/0109
**BASIC NHS Cost** £2.04

## Clinitar Gel

**PRESENTATION** A clear brown gel with a slightly greenish fluorescence, containing Stantar 2.5%
**USES** For the treatment of psoriasis of the scalp and of the body. Other dermatoses responding to tar treatment, such as seborrhoeic dermatitis and chronic eczema.
**DOSAGE AND ADMINISTRATION** Apply to the affected areas once or twice daily.
**CONTRA-INDICATIONS AND WARNINGS** The use of Clinitar gel is not recommended in generalised pustular psoriasis, infections of the skin, or for patients allergic to coal tar. Coal tar can sensitise the skin to U.V. light, leading to sunburn. Clinitar gel should therefore be used cautiously in the sun. Before any irradiation treatment Clinitar gel should be removed completely. During the summer, it is advisable to apply Clinitar gel only once a day at bedtime followed by a bath the next morning. Simultaneous treatments with a coal tar product and drugs which may provoke photosensitivity may create a potential risk for the patient to acquire unintended sunburn on exposure to the sun or sunlight from an artificial source
**PHARMACEUTICAL PRECAUTIONS** None
**LEGAL CATEGORY** P
**PACKAGE QUANTITIES** 40g tube
**FURTHER INFORMATION** Clinitar gel contains Stantar, which is an extract of coal tar standardised to have a photosensitising activity corresponding to that of crude coal tar. By using Stantar instead of crude coal tar a product is obtained which is easy to apply, has no unpleasant smell, and is cosmetically inconspicuous
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***Occasional Review***

# Hyperlipidaemia due to isotretinoin and etretinate: possible mechanisms and consequences

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Recent discussion of the adverse effects of isotretinoin and etretinate has focussed on teratogenicity (Chen, 1985) and unwanted effects on bone (McGuire, Milstone & Lawson, 1985; Burge & Ryan, 1985). However, there is now sufficient information on the adverse effects of these retinoids on serum lipids to suggest possible mechanisms and consequences.

Significant increases in mean levels of both serum triglyceride and cholesterol have been found after treatment with isotretinoin (Lyons, *et al.*, 1982; Katz, Jorgensen & Nigra, 1980; Zech *et al.*, 1983; Marsden *et al.*, 1984; Marsden, Laker & Shuster, 1985a; Vahlquist *et al.*, 1985a; Gollnick *et al.*, 1985). The increases are proportional to the dose of the drug (Marsden *et al.*, 1985a; Katz *et al.*, 1980; van der Schroeff & Jansen, 1985) but are not related to pre-treatment levels of triglyceride or cholesterol (Marsden *et al.*, 1985a; Vahlquist *et al.*, 1985a). They persist throughout treatment and reverse within 4 weeks of stopping isotretinoin (Lyons *et al.*, 1982; Marsden *et al.*, 1984). Ultracentrifugation studies to separate lipoprotein fractions show that the largest absolute increase in triglyceride is in the fraction associated with very low density lipoprotein (VLDL), with smaller increases in low density lipoprotein (LDL)-triglyceride and high density lipoprotein (HDL)-triglyceride (Marsden *et al.*, 1985a; Vahlquist *et al.*, 1985a; Gollnick *et al.*, 1985). In contrast, the largest increase in cholesterol is in the fraction associated with LDL, with a small increase in VLDL cholesterol and a decrease in HDL cholesterol (Zech *et al.*, 1983; Marsden *et al.*, 1985a; Vahlquist *et al.*, 1985a; Gollnick *et al.*, 1985). The increases in VLDL and LDL have been confirmed by lipoprotein electrophoresis (Lyons *et al.*, 1982; Gollnick *et al.*, 1985). The structure of lipoproteins is dependent on their apoprotein constituents which form an interface between the non-polar lipid core (triglyceride and cholesterol esters) and the outer layer (phospholipid and free cholesterol). Apoprotein B (apo B) is the major apoprotein in VLDL and is the only apoprotein in LDL; concentrations of apo B are significantly increased after isotretinoin (Vahlquist *et al.*, 1985a; Gollnick *et al.*, 1985). The only other apoprotein measured before and after isotretinoin is apo A, which is a major apoprotein in HDL; a small increase in total apo A in one group of patients was not confirmed in another (Gollnick *et al.*, 1985) and levels of apo $A_I$ and $A_{II}$ were unchanged (Vahlquist *et al.*, 1985a).

Measurements of serum lipids after treatment with etretinate show changes very like those described with isotretinoin. There are increases in mean levels of both triglyceride and cholesterol (Michaëlsson *et al.*, 1981; Ellis *et al.*, 1982; Vahlquist *et al.*, 1985a; Zech *et al.*, 1981; Gollnick *et al.*, 1985). The increases in triglyceride levels are related to the dose of etretinate in some studies (Ellis *et al.*, 1982; Gollnick, 1981) but not in others (Gollnick *et al.*, 1985) and the precise dose response relationship is unclear; comparisons between groups matched for age, sex and pre-treatment lipid values are required. As with isotretinoin, pre-treatment levels of triglyceride and cholesterol do not predict the size of the subsequent increase (Vahlquist *et al.*, 1985; Ellis *et al.*, 1982). Levels of triglyceride return to pre-treatment values by 4 weeks after stopping etretinate (Ellis *et al.*, 1982) and levels of cholesterol by 8 weeks (Vahlquist *et al.*, 1985a). Ultracentrifugation studies show that, as with isotretinoin, the largest absolute increase in triglyceride is in VLDL, with smaller increases in LDL-triglyceride; HDL-triglyceride does not change (Michaëlsson *et al.*, 1981; Vahlquist *et al.*, 1985a; Gollnick *et al.*, 1985). However, findings conflict regarding the proportion of cholesterol in the three lipoprotein fractions after etretinate, with increases in VLDL cholesterol but not in LDL cholesterol (Michaëlsson *et al.*, 1981; Gollnick *et al.*, 1985) or vice versa (Vahlquist *et al.*, 1985a). The same studies have shown that HDL cholesterol is decreased slightly by etretinate. Zech *et al.* (1981) also found decreased HDL cholesterol but no difference was found by Ellis *et al.* (1982). Increased levels of VLDL and LDL have also been found on lipoprotein electrophoresis (Gollnick *et al.*, 1985). The former three studies also describe increases in apo B after etretinate. However, Michaëlsson *et al.* (1980) and Vablquist *et al.* (1985a) found no change in levels of apo $A_I$ and $A_{II}$ after etretinate, while Gollnick *et al.* (1985) found an increase in total apo A. Thus, the changes in lipids and lipoproteins after etretinate are not quite as well characterized as after isotretinoin, and this may be partly because they are smaller (Vahlquist *et al.*, 1985a). There is no convincing evidence that increasing cumulative doses of isotretinoin or etretinate result in increasing lipid levels after the first few weeks of treatment (Marsden *et al.*, 1984, 1985a; Ellis *et al.*, 1982), nor that the prolonged elimination half-life of etretinate delays reversal of hyperlipidaemia.

Because of the similarities in the changes in serum lipids, it is easier to consider both drugs together when discussing the possible mechanisms. Firstly, it is most unlikely that the changes in serum lipids are due to increased absorption of dietary fat as this would result in increased levels of chylomicrons, the lipoprotein particles assembled in the small intestinal mucosal cells and secreted into the blood via lymphatics. However, hyperlipidaemia is present in patients without lipaemic chylomicron-rich serum even after a 14-h fast, and so is independent of fat intake. Moreover, increased chylomicrons have not been found on lipoprotein electrophoresis (Lyons *et al.*, 1982; Marsden & Laker, unpublished observations). Therefore, decreased catabolism of chylomicrons is also unlikely, and activity of heparin-stimulated serum lipoprotein lipase (LPL), the enzyme responsible for intravascular degradation of chylomicrons at the vascular endothelium, is no different after isotretinoin (van der Schroeff & Jansen, 1985). LPL activity in adipose tissue is also no different after isotretinoin or etretinate, although there is a small and unexplained decrease in skeletal muscle LPL activity (Vahlquist *et al.*, 1985b). Although the rate of clearance of an intravenous bolus of Intralipid (medium chain length triglyceride) is reduced after isotretinoin (Vahlquist *et al.*, 1985b; Gollnick *et al.*, 1985) and etretinate (Vahlquist *et al.*, 1985b), the reason for this and its relevance to chylomicron clearance (Carlson & Rössner, 1972) is uncertain.

The third possibility is that there is increased synthesis of triglyceride and cholesterol in the liver. There is no direct evidence for this in man; however, retinoic acid produces increased synthesis of triglyceride in isolated rat liver cells (although cholesterol synthesis is decreased)

(Erdman, Elliott & Lachance, 1977), and hepatic triglyceride secretion increases *in vivo* (Gerber & Erdman, 1981). An increase in hepatic triglyceride synthesis requires an increase in concentration of precursor free fatty acids (FFA). These are unlikely to come from increased adipose tissue lipolysis, as retinoic acid-induced hypertriglyceridaemia in the rat occurs despite adrenalectomy and unchanged plasma FFA levels; in man, plasma FFA levels are lower than normal after isotretinoin (Gollnick *et al.*, 1985). There is no explanation so far for the increased concentrations of FFA, mono- and diglyceride in rat hepatocytes (Erdman *et al.*, 1977); it is unlikely to result from inhibition of hepatic FFA catabolism by $\beta$-oxidation as neither isotretinoin nor etretinate significantly reduces this (Sherratt, personal communication) and triglyceride precursors are probably derived from other sources (Gerber & Erdman, 1982). The possibility that hepatic triglyceride synthesis might be increased after retinoids in man deserves further investigation. Triglyceride synthesis is increased in familial hyper-triglyceridaemia (Brunzell *et al.*, 1977) and, as with isotretinoin and etretinate, results in increased VLDL and LDL triglyceride and decreased HDL cholesterol (Brunzell *et al.*, 1983). However, there is no change in mean levels of LDL cholesterol and so it is unlikely that this is the sole explanation.

The fourth possibility is increased synthesis or decreased catabolism of VLDL and LDL. VLDL particles are assembled in and secreted by the liver; newly secreted VLDL consists of an outer surface of apo B and apo E, cholesterol and phospholipid, and a large inner core of triglyceride. Apo $C_{II}$ is acquired by VLDL from HDL in the circulation, and activates lipoprotein lipase in the vascular endothelium of tissues which utilize triglyceride. As triglyceride is hydrolysed to FFA it is removed from the core of the VLDL particle. Smaller IDL (intermediate density lipoproteins) are produed which are either cleared by the liver via IDL receptor mediated uptake or catabolized further to produce LDL. The turnover of apo B in VLDL and LDL is normally identical, suggesting that all LDL originates from VLDL. The level of apo B is increased by both isotretinoin and etretinate which suggests an increase in the number of VLDL and LDL particles rather than simply an increase in their size due to a larger content of lipid. Therefore, VLDL and LDL synthesis could be increased. The evidence in support of this idea is indirect. Firstly, increased VLDL production would not be expected to alter its lipid composition, which is no different after isotretinoin, and increases in VLDL and LDL triglyceride are related, as might be expected if they had a common cause (Marsden *et al.*, 1985a). Secondly, there are similarities between the changes in serum lipids after isotretinoin and etretinate and those found in familial combined hyperlipidaemia, in which VLDL and LDL triglyceride and cholesterol are increased, HDL triglyceride is increased and HDL cholesterol is decreased (Brunzell *et al.*, 1983). This disease is probably due to defective regulation of apo B synthesis, with increases in apo B in both VLDL and LDL (Chait, Albers & Brunzell, 1980; Brunzell *et al.*, 1983). Thirdly, phenobarbitone increases apo B in VLDL and LDL, and increases serum cholesterol (Durrington, 1979) although changes in triglyceride levels are small (Miller & Nestel, 1973) and, unlike the retinoids, HDL cholesterol is increased; the ratio of triglyceride to cholesterol in LDL increases as with isotretinoin (Marsden *et al.*, 1985a). Because other drugs which increase serum cholesterol also induce hepatic microsomal enzymes, it has been suggested that isotretinoin might have this effect (Daneshmend & Paisey, 1983) but more recent findings do not support this idea (Finnen & Shuster, 1984; Marsden, Shuster & Dennis, 1985b). Further characterization of the increase in apo B levels after retinoids is required; for instance overproduction of VLDL apo B does not necessarily result in increased values for LDL apo B (Eaton, Allen & Schade, 1983), and small increases in apo B may occur in response to increased synthesis of triglyceride in the liver as in familial hypertriglyceridaemia (Brunzell *et al.*, 1983).

The alternative to increased synthesis of VLDL and LDL is decreased catabolism, but this is less likely for the following reasons. As with chylomicrons, removal of triglyceride from VLDL is dependent on lipoprotein lipase, and the majority of the evidence shows that this is no different after treatment with retinoids. Although isotretinoin and etretinate bind to VLDL (Zech *et al.*, 1981) this does not seem to affect its suitability as a substrate for LPL (van der Schroeff & Jansen, 1985). Furthermore, there is no increase in the proportion of triglyceride in VLDL as would be expected with decreased degradation, and no accumulation of partially catabolized VLDL or IDL remnants on lipoprotein electrophoresis (Marsden & Laker, unpublished observations). Reduced catabolism of VLDL and IDL due to impaired clearance by the liver in familial type 3 hyperlipoproteinaemia results in low levels of LDL, in contrast to the increased values found after isotretinoin and etretinate. This is probably because part of the conversion of IDL to LDL occurs in the liver (Brown, Goldstein & Fredickson, 1983). Conversely, although catabolism of LDL is grossly impaired in familial hypercholesterolaemia, levels of triglyceride and VLDL cholesterol are usually not increased (Khachadurian & Uthman, 1973).

Finally, the explanation for the decrease in HDL cholesterol may simply be that it is a result of its inverse relationship with VLDL triglyceride (Barter & Conner, 1975). The reason for this relationship is probably that as VLDL is catabolized, apo C is released and taken up by HDL; there is evidence that this indirect relationship persists during treatment with isotretinoin (Marsden *et al.*, 1985a).

The known major risks of hyperlipidaemia are pancreatitis with massive hypertriglyceridaemia and increased atheroma and ischaemic heart disease (IHD). Pancreatitis has not been described as a consequence of retinoid treatment and is only likely at levels of triglyceride greater than 10 mmol/l. Levels of this order have been found during treatment with isotretinoin (Lyons *et al.*, 1982; Ellis, 1982; Dicken & Conolly, 1980; Katz *et al.*, 1980) and these patients had increased pre-treatment levels. There is evidence for similarly large increases in serum triglyceride in patients with an underlying primary disorder of triglyceride metabolism who are subsequently exposed to one of the secondary causes of hypertriglyceridaemia such as diabetes or alcohol (Chait & Brunzell, 1983) and this suggests a synergistic effect. In contrast, patients with secondary hypertriglyceridaemia due to diabetes, obesity or alcohol who are treated with etretinate seem to have much smaller increases with triglyceride levels usually less than 3·5–4·0 mmol/l (Gollnick *et al.*, 1985). This suggests an additive effect. However, the absolute increase in cholesterol does not appear to be larger in these subjects.

There is conflicting evidence as to whether triglyceride levels correlate with the risk of IHD independently of other variables (Hulley *et al.*, 1980). However, recent evidence shows that LDL apo B is increased in a subgroup with hypertriglyceridaemia but normal levels of serum cholesterol, and it is these individuals who have increased risk of atherosclerosis (Sniderman *et al.*, 1982). Moreover, apo B concentrations correlate better with proven IHD than does serum cholesterol (Whayne *et al.*, 1981). As already discussed, total apo B, and presumably LDL apo B, is increased after isotretinoin and etretinate. Although the relationship between total serum cholesterol and IHD is well established, it reflects the opposing and independent influences of LDL cholesterol, which is directly related to IHD, and HDL cholesterol, which is indirectly related (Kannel, Castelli & Gordon, 1979). Both isotretinoin and etretinate increase LDL cholesterol and decrease HDL cholesterol. Therefore, these drugs adversely affect three important predictors of risk for IHD. The similarity of the pattern of the changes in serum lipids to that in familial combined hyperlipidaemia might also be relevant because of the increased risk of IHD associated with this disease.

It is not possible to make accurate predictions about the size of any change in risk of IHD; however, a normal 35-year-old man with a serum cholesterol of 6·0 mmol/l has a 60% greater risk of a coronary in the following 6 years than if his cholesterol level was 5·2 mmol/l (Kannel, 1972). Increases of about 1·0 mmol/l are frequently found with isotretinoin (Marsden *et al.*, 1985a; Lyons *et al.*, 1982; Vahlquist *et al.*, 1985a) and are not uncommon with etretinate (Gollnick *et al.*, 1985). A crucial point is that these values fall well below the upper limit of the reference range for cholesterol of about 7·1 mmol/l which merely denotes the 95th centile. There is now strong evidence that the relationship between total serum cholesterol and risk of IHD is continuous, and valid throughout the range of cholesterol concentrations; so much so that a man aged 30–39 with a level above 6·2 mmol/l is judged to be at high risk (Steinberg, 1985). Therefore, simply classifying a cholesterol value as normal or abnormal is misleading; increased risk does not suddenly begin above the upper limit of the reference range.

So what can be done to minimize these problems? It seems wise to treat patients with pre-existing hypertriglyceridaemia with care as the subsequent increase may be large enough to cause pancreatitis; treatment should probably be stopped if values rise much above 5·0 mmol/l. Reduction of dose is likely to reduce levels of both triglyceride and cholesterol, although the size of this effect is unpredictable from existing data. If treatment is for more than a few months it may be worth making dietary changes. Reduction of total calorie intake is effective in reducing triglyceride, and sometimes cholesterol levels, in obese patients. In the non-obese, total calories should remain unchanged but total dietary fat should be reduced to 25–30% of calories and the resulting calorie deficit replaced by carbohydrate. Cholesterol should be reduced to 250 mg per day or less and polyunsaturated fat increased to 10% of calories. Reduction of a high alcohol intake may produce large decreases in triglyceride levels. These changes in diet should normally be suitable for all ages over 2 years (Steinberg, 1985). There is no information about the efficacy or safety of hypolipidaemic drugs in the treatment of retinoid-induced hyperlipidaemia.

In conclusion, it is clear that both isotretinoin and etretinate increase mean levels of serum triglyceride and cholesterol. The changes in VLDL and LDL suggest that the increases may be due to enhanced synthesis of lipoproteins, and higher levels of apo B after treatment with retinoids supports this idea; increased lipid synthesis is another possibility. Kinetic studies are now required to measure synthesis of lipid in the liver, and lipoprotein turnover and composition. Because of the increases in apo B and LDL cholesterol and decreased HDL cholesterol it is likely that long-term use of both drugs will result in accelerated atherogenesis and consequently increased risk of ischaemic heart disease.

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***Clinical and Laboratory Investigations***

# Epidermal hyperproliferation following the induction of microabscesses by leukotriene $B_4$

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## SUMMARY

The percentage of non-diploid epidermal cells was determined by flow cytometry following application of leukotriene $B_4$ ($LTB_4$) to human skin. Doses in the range 35–500 ng were shown to cause a marked increase in proliferation, the non-diploid cells reaching a maximum between 72 and 96 h after $LTB_4$ application. No difference was observed between the response of healthy controls and the uninvolved skin of psoriatic patients. We suggest, therefore, that the hyperproliferation is a consequence of the physical disruption of the stratum corneum accompanying the rupture of microabscesses.

The penetration of neutrophil polymorphonuclear leukocytes (NF) into the epidermis is an early and characteristic feature of the psoriatic lesion. However, the possibility of a causal relationship between this phenomenon and the subsequent hyperproliferation has never been tested experimentally.

It has been shown recently that topical application of leukotriene $B_4$ ($LTB_4$) results in a dose-dependent induction of microabscesses (Camp *et al.*, 1984; van de Kerkhof, Bauer & Maassen-de Grood, 1985). The well-defined time-phasing of this phenomenon permits the study of subsequent changes in growth control. Here we report flow cytometric measurement of the percentage of non-diploid (S and $G_2M$) cells in biopsies following the application of $LTB_4$ to human skin.

## METHODS

Eight healthy controls, without any history or sign of psoriasis, and seven patients with stable plaque psoriasis, untreated for at least one month, participated in these experiments. Prior approval of the Medical Ethics Committee was obtained.

$LTB_4$ applications were performed as described previously (van de Kerkhof *et al.*, 1985). In brief, doses of 10, 35, 137 or 500 ng $LTB_4$ in ethanol were applied through glass cylinders

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(diameter 5·5 mm) to the clinically normal skin of the back. The ethanol was evaporated with a stream of nitrogen. The test sites were covered with impermeable dressings for 24 h. At different time intervals biopsies, approximately 3 mm diameter and 0·3 mm thickness, were taken free-hand using a razor-blade in conjunction with a metal guard (Bauer & de Grood, 1976). No anaesthetic agent was employed.

Biopsies for histological studies were processed for paraffin sectioning and haematoxylin-eosin staining. Those for investigation of cell cycle kinetics were trypsinized, stained with propidium iodide and analysed by flow cytometry using techniques which have already been described in full (Bauer & Boezeman, 1983).

## RESULTS

The clinical response of psoriatic patients to $LTB_4$ was similar to that of healthy controls. In general, 24 h after the application of 500 ng only a faint erythema was seen. After 36 h there was pronounced erythema often exceeding the wells of the cylinder with a variable degree of oedema. At this time a slight scaling began which was most marked at 48–96 h following $LTB_4$ application.

The response of patients and controls were also similar at the histological level. Typical changes are illustrated in Figure 1. After 24 h a heavy NF infiltrate is seen with medium to large microabscesses (Fig. 1a). By 36 h there is a patchy disruption of the stratum corneum (Figs 1b and 1c). At 48 h this process is complete, with total desquamation of the stratum corneum; the stratum Malphigii appears normal (Fig. 1d).

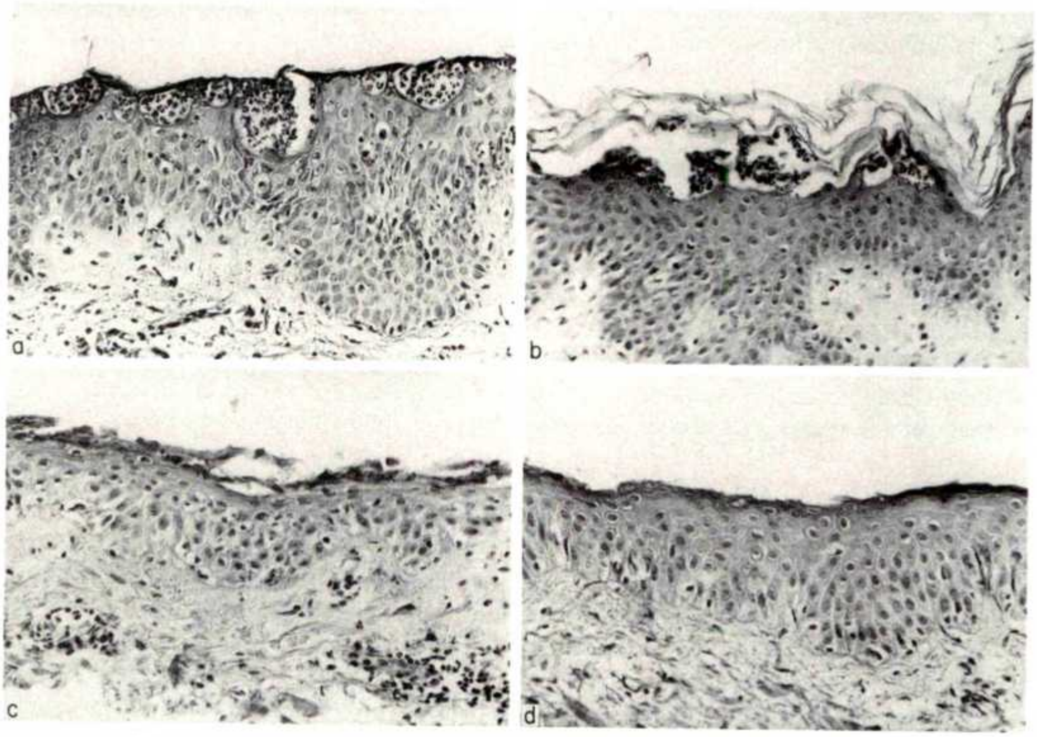

FIGURE 1. Histological appearance of the epidermis at different times following the application of 500 ng $LTB_4$. (a) 24 h; (b) and (c) 36 hours; (d) 48 h.

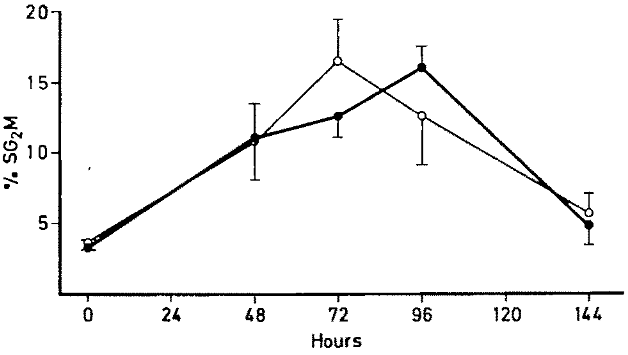

FIGURE 2. Percentages of non-diploid cells (mean ± SEM) at different times following the application of 500 ng $LTB_4$ in healthy controls, ○, and psoriatic patients, ●. Number of patients and controls tested after each time interval:

| Time (h) | 0 | 48 | 72 | 96 | 144 |
|---|---|---|---|---|---|
| No. of patients | 6 | 6 | 7 | 7 | 5 |
| No. of controls | 6 | 8 | 8 | 8 | 6. |

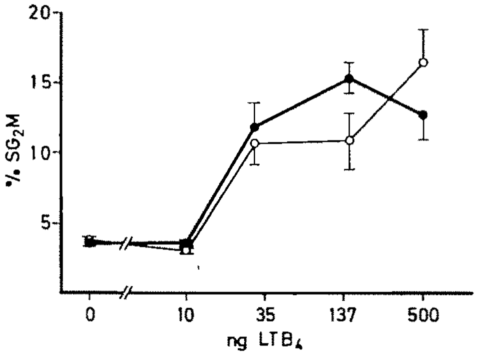

FIGURE 3. Percentages of non-diploid cells measured 72 h after different doses of $LTB_4$ in healthy controls, ○, and psoriatic patients, ●. Numbers of patients and controls tested with each dose of $LTB_4$:

| $LTB_4$ (ng) | 0 | 10 | 35 | 137 | 500 |
|---|---|---|---|---|---|
| No. of patients | 6 | 4 | 5 | 5 | 7 |
| No. of controls | 6 | 4 | 6 | 6 | 8. |

Percentages of non-diploid (S and $G_2M$) keratinocytes at different times after the application of 500 ng $LTB_4$ are shown in Figure 2. A broad peak is seen between 72 and 96 h, the number of non-diploid cells rising to about 5 times the normal level. A dose-response curve, assessed 72 h after application of $LTB_4$, is illustrated in Figure 3. It is seen that there is no response to the lowest dose employed (10 ng), whereas at 35 ng stimulation is already approaching maximal. There was no significant difference between the data for psoriatic patients and controls in either the time-curve or dose-response curve.

## DISCUSSION

This investigation has shown that application of $LTB_4$ to human skin induces a proliferative response. Several explanations are possible. Firstly, it may be a direct effect of $LTB_4$ on the germinative cell population; a stimulation of mitotic activity of cultured keratinocytes has been reported by Kragballe and Voorhees (1984). Secondly, it may be caused by the release of mediators such as proteases (Lazarus & Gilgor, 1979) from the infiltrating NF. Finally, it could be a regenerative response initiated by the loss of the stratum corneum, i.e. a 'stripping' from the inside.

The time-response curve (Fig. 2) suggests that the last of these alternatives is the most likely. It is well established that procedures such as sellotape stripping result in the mobilization of a cohort of $G_0$ cells (Gelfant, 1982). These enter the cell cycle and appear maximally in S and $G_2M$ phases about 40 h after the injury (Bauer & Boezeman, 1983; Bauer, *et al.*, 1985). Thus, we would expect that the immediate stimulus for $G_0$ mobilization is an event occurring about 40 h prior to the appearance of non-diploid cells in the germinative population, i.e. about 32–56 h after $LTB_4$ application. As the histology shows (Fig. 1), this corresponds rather well with the period during which the stratum corneum is lost. Such a concept is also compatible with the dose-response curve; after a dose of 10 ng $LTB_4$ the infiltrate consists mainly of isolated NF and the stratum corneum remains intact (van de Kerkhof *et al.*, 1985).

The question of whether this sequence of events is of significance in the pathogenesis of the psoriatic lesion thus remains open, but the relative sparcity of microabscesses in chronic plaque psoriasis suggests that this is unlikely. It may nevertheless act as an endogenous 'Koebner response', linking the initial pustule formation with the subsequent hyperproliferation in the pustular form of this disease.

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# Topical PUVA treatment increases epidermal beta-adrenergic adenylate cyclase responsiveness

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## SUMMARY

The effects of topical PUVA treatment on the epidermal cyclic AMP system were investigated. 8-methoxypsoralen (8-MOP), 0·3% in ethanol was applied to the backs of pigs which were then irradiated with UVA. A significant increase in the epidermal beta-adrenergic adenylate cyclase response was observed 24 h after low (1·1 J/cm$^2$) and moderate (2·1 J/cm$^2$) dose irradiation. There was no significant change in the adenosine- or histamine-mediated adenylate cyclase responses. 8-MOP application or UVA irradiation alone had no effect on the beta-adrenergic adenylate cyclase response. PUVA treatment with a higher irradiation dose (4·2 J/cm$^2$) produced no increase in the beta-adrenergic response and adenosine- and histamine-mediated adenylate cyclase responses were decreased. Cyclic AMP phosphodiesterase activity was decreased by PUVA treatments using UVA doses of 1·1 and 2·1 J/cm$^2$; however, the change was not statistically significant. The increased beta-adrenergic response was also observed in the presence of the cyclic AMP phosphodiesterase inhibitor, isobutylmethylxanthine. These results indicate that epidermal adenylate cyclase responsiveness is affected by topical PUVA treatment *in vivo*.

A defective beta-adrenergic adenylate cyclase response has been observed in hyperproliferative conditions of keratinocytes including psoriasis (Iizuka *et al.*, 1978), and might be associated with the pathophysiology of this condition. Using an *in vitro* pig skin incubation system we have shown that drugs which are effective in treating psoriasis (such as glucocorticoids and retinoids) increase the beta-adrenergic response of the epidermis (Iizuka & Ohkawara, 1983; Iizuka, Ohkuma & Ohkawara, 1985). Subsequently, we have found that UVB irradiation, an essential constituent of the Goeckerman regimen (Le Vine, White & Parrish, 1979), also increases the beta-adrenergic response of epidermis *in vivo* (Iizuka, Kajita & Ohkawara, 1985). Therefore, we suggested that the increase in the beta-adrenergic response might be relevant to the therapeutic effect of these agents on the hyperproliferative epidermis of psoriasis. PUVA treatment has been

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shown to be a useful treatment for psoriasis. We have investigated the effect of PUVA treatment on the beta-adrenergic response of epidermis *in vivo*.

## METHODS

### *Psoralen*

A 1% 8-methoxypsoralen (8-MOP) solution was purchased from Taisho Pharmaceutical Co. (Tokyo, Japan). This was diluted in ethanol to give a 0·3% solution.

### *Ultraviolet light source*

For UVA irradiation, a Toshiba-Eisai Dermaray machine (M-DMR-1 Tokyo, Japan) was used with ten 20 watt fluorescent black light lamps (FL-20-BLB, Toshiba, Tokyo, Japan). These lamps emit wavelengths of 300–430 nm with the main emission between 347–357 nm and peak emission at 352 nm. The power output of this instrument was 7·0 mW/cm$^2$ with a target distance of 15 cm, as measured by a UV radiometer (Tokyokogaku, Tokyo, Japan).

### *PUVA treatment*

Domestic white-haired pigs weighing 5–10 kg were anaesthetized with Nembutal (Dainippon, Osaka, Japan) intra-peritoneally (dose 30 mg/kg). After clipping the hair on the back and washing the skin surface, 0·3% 8-MOP solution was applied to a 25 cm$^2$ area of skin (approximately 10 $\mu$l/cm$^2$). Twenty minutes after 8-MOP application, the skin was irradiated with UVA at a dosage of 1·1, 2·1, or 4·2 J/cm$^2$, (corresponding to 2·5, 5, and 10 min irradiation respectively). Twenty-four hours later, the pigs were re-anaesthetized and 0·3 mm skin biopsies were obtained from PUVA-treated and from untreated sites using a Castroviejo keratotome (Storz Instrument Co., St. Louis, U.S.A.). Since variations in the adenylate cyclase response have been found among different pigs (Iizuka, Kishiyama & Ohkawara, 1983), comparisons were made between PUVA-treated and untreated skin from the same pig, obtained on the same occasion. Each experimental series (using the three test reagents and a control) was performed at least three times using different pigs. Control skin specimens were also obtained from sites treated with UVA irradiation only and 8-MOP only.

### *Cyclic AMP production and phosphodiesterase activity*

The skin specimens were cut into 5 × 5 mm squares, washed three times in RPMI 1640 medium, pH 7·0, and pre-incubated in the same medium for 15 min at 37°C to standardize the cyclic AMP level (Yoshikawa *et al.*, 1975). After pre-incubation, two squares were randomly selected and floated with their keratin layers uppermost in RPMI 1640 containing either epinephrine 50 $\mu$mol/l, adenosine 2 mmol/l, or histamine 1 mmol/l. It has been shown that these concentrations are sufficient to give maximal accumulation of cyclic AMP after 5 min incubation without cyclic AMP phosphodiesterase inhibitors (Adachi, 1977). After 5 min incubation in a water bath at 37°C, the skin squares were rapidly frozen between two blocks of dry ice. After partial purification, using the method of Yoshikawa *et al.* (1975), the cyclic AMP content in the skin squares was measured by radioimmunoassay using a Yamasa cyclic AMP assay kit (Yamasa Shoyu Co., Tokyo, Japan).

The cyclic AMP phosphodiesterase activity in skin squares was measured by the method of Adachi *et al.* (1976) using cyclic AMP as the substrate, at concentrations of 0·75 $\mu$mol/l and 102 $\mu$mol/l for low and high Km enzymes respectively. Protein concentrations were measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Statistical analysis of the data was performed using Student's *t*-test.

## RESULTS

Table 1 shows the adenylate cyclase activity 24 h after PUVA treatment. At low (1·1 J/cm$^2$) and moderate (2·1 J/cm$^2$) doses of irradiation, PUVA produced a significant increase in the beta-

TABLE 1. Effects of PUVA treatment on the adenylate cyclase activity of pig epidermis

| UVA dosage (J/cm$^2$) | Incubation | Cyclic AMP (pmol/mg protein) | | |
|---|---|---|---|---|
| | | Control skin | PUVA treated skin | $P$ |
| 1·1 | No addition | 1·4 ± 0·1 | 1·1 ± 0·1 | NS |
| " | Epinephrine | 5·3 ± 0·7 | 24·9 ± 3·7 | <0·01 |
| " | Adenosine | 36·6 ± 4·3 | 33·9 ± 3·8 | NS |
| " | Histamine | 35·0 ± 4·1 | 34·8 ± 0·7 | NS |
| 2·1 | No addition | 1·1 ± 0·1 | 1·6 ± 0·2 | NS |
| " | Epinephrine | 3·6 ± 0·1 | 18·2 ± 2·5 | <0·01 |
| " | Adenosine | 27·5 ± 2·2 | 26·6 ± 1·8 | NS |
| " | Histamine | 23·8 ± 3·8 | 27·6 ± 4·7 | NS |
| 4·2 | No addition | 1·6 ± 0·2 | 1·1 ± 0·2 | NS |
| " | Epinephrine | 2·5 ± 0·2 | 2·2 ± 0·2 | NS |
| " | Adenosine | 21·4 ± 0·5 | 7·4 ± 0·9 | <0·01 |
| " | Histamine | 12·0 ± 0·8 | 5·8 ± 0·5 | <0·01 |

Figures are means ± SE of four separate experiments on the same pig.

TABLE 2. Comparison of the effects of PUVA treatment with the effects of UVA irradiation and 8-MOP application alone on the beta-adrenergic responses of pig epidermis

| | Cyclic AMP (pmol/mg protein) | | | |
|---|---|---|---|---|
| | Control | UVA | 8-MOP | PUVA |
| Epinephrine | 4·4 ± 0·9 | 5·2 ± 0·6* | 5·4 ± 0·4* | 17·3 ± 1·9** |

UVA dosage 1·1 J/cm$^2$.

Figures are means ± SE of four separate experiments on the same pig.

* $P<0·01$ compared with PUVA treatment, but not significantly different from control.

** $P<0·01$ compared with control.

Basal levels of cyclic AMP were approximately 1·0 pmol/mg protein and showed no significant difference.

adrenergic adenylate cyclase activity of the epidermis ($P < 0{\cdot}01$), but had no significant effect on either the adenosine- or histamine-mediated adenylate cyclase activity. At the highest irradiation dose (4·2 J/cm$^2$) PUVA produced no significant change in the beta-adrenergic response but caused significant decrease in the adenosine- and histamine-mediated adenylate cyclase activity ($P < 0{\cdot}01$). Neither UVA irradiation nor 8-MOP application alone had any effect on the beta-adrenergic response (Table 2).

The effect of PUVA treatment on the cyclic AMP phosphodiesterase activities in the epidermis is shown in Table 3. There was no difference in low Km phosphodiesterase activity between control and PUVA-treated skin. High Km enzyme activity was decreased by PUVA treatment; however, the difference was not statistically significant.

An increase in beta-adrenergic adenylate cyclase activity was observed in the presence of the cyclic AMP phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX) 1 mmol/l (Table 4). The effect of PUVA treatment was less when measured in the presence of IBMX. The addition of IBMX resulted in a 5·2-fold increase in cyclic AMP levels in control skin incubated with epinephrine, compared with a 2·0-fold increase in PUVA-treated skin.

TABLE 3. Effects of PUVA treatment on the cyclic AMP phosphodiesterase activity of pig epidermis

| | Phosphodiesterase activity (pmol/min/mg protein) | |
|---|---|---|
| Enzyme type | Control skin | PUVA treated skin |
| Low Km | 15·4±0·6 | 15·4±0·3 |
| High Km | 471·4±33·4 | 374·4±35·0 |

UVA dose 2·1 J/cm$^2$.

Figures are means ± SE of four separate experiments on the same pig. Similar results were obtained with higher and lower doses of UVA.

TABLE 4. Effect of the cyclic AMP phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX) on the beta-adrenergic response of pig epidermis

| | Cyclic AMP (pmol/mg protein) | | |
|---|---|---|---|
| | Control skin | PUVA treated skin | *P* |
| Epinephrine | 4·4±0·9 | 17·3±1·9 | <0·01 |
| Epinephrine + IBMX | 22·8±1·9 | 34·3±1·6 | <0·01 |

UVA dose 1·1 J/cm$^2$.

Figures are means ± SE of four separate experiments on the same pig. Basal levels of cyclic AMP were approximately 1·0 pmol/mg protein and showed no significant difference.

## DISCUSSION

Our results indicate that topical PUVA treatment *in vivo* increases epidermal beta-adrenergic adenylate cyclase activity. This effect was specific to the beta-adrenergic system and was essentially similar to the effect of UVB irradiation described previously (Iizuka *et al.*, 1985a).

Hormone-induced cyclic AMP accumulation is determined by both adenylate cyclase and cyclic AMP phosphodiesterase activity. In the present study, PUVA treatment produced a decrease in high Km cyclic AMP phosphodiesterase activity. Since the effect of PUVA was specifically on the beta-adrenergic receptor, and since this effect was also observed in the presence of the cyclic AMP phosphodiesterase inhibitor, IBMX, it seems that PUVA treatment specifically affected the beta-adrenergic adenylate cyclase system of epidermis. The difference in the beta-adrenergic response produced by PUVA, was, however, less marked when measured in the presence of IBMX. This would indicate that the effect of PUVA treatment on the beta-adrenergic response is mediated through alterations in both beta-adrenergic adenylate cyclase activity and cyclic AMP phosphodiesterase activity, although the change produced in phosphodiesterase activity was not statistically significant. The results with high dose PUVA suggest a toxic effect on the keratinocytes as has been found with UVB irradiation (Iizuka *et al.*, 1985a).

It has been established that PUVA treatment suppresses epidermal DNA synthesis both *in vitro* (Walter *et al.*, 1973) and *in vivo* (Epstein & Fukuyama, 1975). Although this effect is usually assumed to explain its clinical efficacy on the hyperproliferative epidermis of psoriasis, the effect of PUVA treatment is not restricted to DNA metabolism in the epidermis. It has been reported that PUVA treatment affects keratinocyte membrane markers and that this change might be due to direct damage of the membrane glycoprotein or other membrane components (Danno, Takigawa & Horio, 1983). Since adenylate cyclase is a membrane-bound enzyme complex, the cell membrane perturbation induced by PUVA could have a profound effect on adenylate cyclase activity. For example, the membrane phospholipid environment is known to be important in the activation of the beta-adrenergic adenylate cyclase system (Ross, 1982).

Recently, we have reported that UVB irradiation increases the beta-adrenergic adenylate cyclase activity of epidermis *in vivo* (Iizuka *et al.*, 1985a). Although UVB irradiation and PUVA treatment have some different effects on DNA, RNA and protein metabolism (Epstein & Fukuyama, 1975; Morrison *et al.*, 1977), both modalities have been used for psoriasis with considerable clinical efficacy (Boer *et al.*, 1984). Our results indicate that both treatments have a similar effect on cyclic nucleotide metabolism. Since a beta-adrenergic defect has been associated with increased epidermal cell proliferation (Iizuka *et al.*, 1978; Solanki & Murray, 1982), and since drugs which have a therapeutic effect on the psoriatic hyperproliferative epidermis (such as glucocorticoids and retinoids) also increase the beta-adrenergic response of the epidermis (Iizuka *et al.*, 1985b), the increased beta-adrenergic responsiveness following PUVA treatment (as well as UVB irradiation) might be related to the therapeutic efficacy of these treatments.

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# Early warning skin signs in AIDS and persistent generalized lymphadenopathy

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## SUMMARY

Distinctive patterns of skin disease other than Kaposi's sarcoma have been seen in patients with AIDS, in others with persistent generalized lymphadenopathy (PGL) and in a group at high risk of developing AIDS. We found a chronic acneiform folliculitis on the face, back, chest and buttocks, extensive cutaneous fungal infections and a striking neck and beard impetigo. These skin diseases were not present in asymptomatic male homosexual control subjects, 32% of whom were found to have antibodies to human T cell lymphotropic virus type III (HTLV-III). We regard these dermatoses as early warning signs of AIDS.

The current epidemic of AIDS has attracted much attention, and the association of immune deficiency, opportunistic infections, and Kaposi's sarcoma is now well known (Gottlieb *et al.*, 1981). The characteristics of the immune deficiency are an impairment of cell-mediated immune responses, depletion of T helper lymphocytes with inversion of the normal helper/suppressor ratio and a polyclonal rise in IgG. The syndrome of persistent generalized lymphadenopathy (PGL) may in some cases progress to AIDS (*Morbidity & Mortality Weekly Report*, 1982). Both conditions occur in male homosexuals, intravenous drug abusers and Haitian immigrants to the U.S.A., but also at risk are recipients of blood products, and the heterosexual partners and children of AIDS patients. The human T lymphotropic virus type III (HTLV-III) has been isolated from the peripheral blood lymphocytes of both PGL and AIDS patients (Gallo *et al.*, 1984) and HTLV-III antibodies have been demonstrated in these groups (Sarngadharan *et al.*, 1984; Cheinsong-Popov *et al.*, 1984) and in the normal healthy homosexual population.

Cutaneous manifestations of AIDS and PGL include Kaposi's sarcoma (Hymes *et al.*, 1981), chronic ulcerative genital herpes simplex (Siegal *et al.*, 1981), herpes zoster and mucocutaneous candidiasis (Gottlieb *et al.*, 1981), fungal infections, chronic pyoderma and, more recently, seborrhoeic dermatitis (Eisenstat & Wormser, 1984). Immune complex vasculitis, folliculitis and xeroderma have recently been added to this list (Farthing, Staughton & Rowland Payne, 1985). We would like to draw attention to some distinctive patterns of skin disease which we

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have seen in patients with AIDS and PGL, and in patients at high risk of AIDS. We have also assessed the prevalence of skin disease and of antibodies to HTLV-III in a population of male homosexuals.

## METHODS

A total of 172 male homosexual patients was studied. These included nine patients with AIDS, 15 with PGL and four at high risk of AIDS. AIDS was defined according to the U.S. Center for Disease Control criteria (*Morbidity & Mortality Weekly Report*, 1982) and PGL by lymphadenopathy for 3 months or more and at two sites other than the inguinal nodes. The high risk group was defined as male homosexual patients with either multiple sexual encounters in the U.S.A., or known sexual contact with an AIDS patient. A group of 144 asymptomatic male homosexuals who were attending the department of genitourinary medicine served as controls.

The prevalence of HTLV-III antibodies was measured in 168 patients using a competitive binding radioimmunoassay (Mortimer *et al.*, 1985). The HTLV-III antibody status was not known to the examiner when the subjects were assessed for skin disease. Six patients with PGL and one high risk patient have since developed AIDS, and nine patients have died from AIDS.

## RESULTS

The skin diseases found in the different groups are listed in Table 1. Three unusual patterns of skin disease that were not seen in the controls were observed in patients with AIDS or PGL and in the high risk group. The first of these was a chronic acneiform folliculitis which was seen in

TABLE 1. Skin diseases in AIDS, PGL and high risk patients and controls

| | No. of patients | | | Controls | |
|---|---|---|---|---|---|
| Skin disease | AIDS | PGL | High risk | HTLV-III positive | HTLV-III negative |
| Kaposi's sarcoma | 6 | 0 | 0 | 0 | 0 |
| Extensive fungus infection | 5 | 6 | 2 | 0 | 0 |
| Mucocutaneous candidiasis | 4 | 0 | 0 | 1 | 0 |
| Beard & neck impetigo | 2 | 0 | 2 | 0 | 0 |
| Axillary folliculitis | 2 | 4 | 2 | 0 | 0 |
| Acneiform folliculitis | 0 | 7 | 3 | 0 | 0 |
| Folliculitis—other minor | 0 | 0 | 0 | 0 | 1 |
| Eczema | 2 | 1 | 0 | 0 | 0 |
| Xeroderma | 2 | 4 | 0 | 0 | 0 |
| Seborrhoeic dermatitis | 1 | 4 | 0 | 4 | 3 |
| Herpes simplex | 0 | 4 | 0 | 0 | 0 |
| Herpes zoster | 0 | 2 | 0 | 0 | 0 |
| Molluscum contagiosum | 2 | 0 | 0 | 0 | 0 |
| Leukoplakia | 0 | 0 | 1 | 0 | 0 |
| Vasculitis | 0 | 0 | 1 | 0 | 0 |
| Pityriasis versicolor | 0 | 0 | 0 | 2 | 0 |
| Psoriasis | 0 | 0 | 0 | 1 | 1 |
| Aphthous ulcers | 0 | 0 | 0 | 0 | 1 |
| Grover's disease | 0 | 0 | 0 | 1 | 0 |
| Total no. of patients | 9 | 15 | 4 | 46 | 98 |

seven patients with PGL (46%), and in three of the high risk group (75%). In its early stages the eruption resembled acne vulgaris and consisted of follicular pustules on the face, back and upper chest. Progression often occurred, with pustules developing on a background of diffuse erythema. Excoriation and eczematization could be marked, especially when lesions developed on the thighs and perineum. An axillary folliculitis was found in four patients (26%) with PGL, two (22%) with AIDS and two (50%) in the high risk category (Fig. 1). Biopsies from the areas of folliculitis on the trunk showed, in all patients, a severe mixed perifollicular infiltrate with polymorphs, lymphocytes and plasma cells. Treatment with flucloxacillin, dilute topical steroids and emollients was initially successful but relapse was frequent.

The second pattern, a florid impetigo, localized to the beard area and across the front of the neck, was seen in two (22%) of the patients with recently diagnosed AIDS and in two (50%) of the patients in the high risk group. Bacterial cultures were performed in six of the 10 patients with folliculitis and in three of the four cases of impetigo. *Staphylococcus aureus* was isolated from all cases of impetigo and from three patients with chronic folliculitis. Normal skin flora only were isolated from the three other cases of folliculitis. Pityrosporum yeasts were seen in one of four skin biopsies from patients with folliculitis. Treatment with topical and systemic antibiotics was successful, but again relapse was common. Thirdly, extensive infection of the toewebs, soles and groins with *Trichophyton rubrum* was present in 13 patients, five in the AIDS group, six in the PGL group and two in the high risk group. One patient had *Epidermophyton floccosum* infection of the toe clefts, soles, groin, trunk, face, finger clefts and nails. Treatment with griseofulvin controlled the *T. rubum* infection in most patients, but was not successful in the *E. floccosum* infection which was also resistant to ketoconazole. Relapse was common on stopping therapy.

All ten patients with chronic acneiform folliculitis have been followed. Seven have so far developed AIDS, and six of these have died. Six of eight patients in the PGL and high risk group who had widespread fungus infection have developed AIDS. Two patients with beard and neck impetigo and AIDS have died. The two other patients with impetigo have been lost to follow up.

Six patients with AIDS had Kaposi's sarcoma. The AIDS patients also displayed a

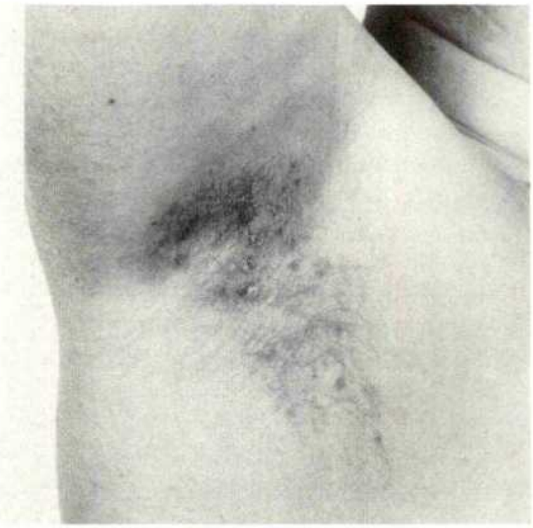

FIGURE 1. Axillary folliculitis.

miscellany of other skin disorders including molluscum contagiosum, herpes simplex, candidiasis, seborrhoeic dermatitis, eczema, leukonychia, leukoplakia and xeroderma. Other skin diseases seen in patients with PGL were herpes zoster, genital and labial herpes simplex, and a florid seborrhoeic dermatitis. One high risk patient presented with a leukocytoclastic vasculitis, in addition to extensive fungal infection and an acneiform and axillary folliculitis. Another had leukoplakia together with a chronic folliculitis. Only five patients with PGL had no skin disease, whilst many of the patients in the PGL, AIDS and high risk groups had three or more separate skin disorders.

In the control group, seborrhoeic dermatitis was seen in four (8·5%) of the seropositive and three (3%) of the seronegative subjects. Tinea versicolor was found in two seropositive controls and oral candidiasis in one. One seropositive patient had transient acantholytic dermatosis (Grover's disease). Folliculitis of the beard area was seen in one seronegative patient. No control subject had acneiform or axillary folliculitis, impetigo of the beard area or extensive fungus infection.

The prevalence of antibodies to HTLV-III is shown in Table 2. All AIDS patients, 13 of 15 PGL patients tested and both of two high risk patients tested were seropositive. Of the 144 control patients 46 (32%) were positive.

## DISCUSSION

Patients with AIDS and AIDS-related disorders are prone to skin disorders other than Kaposi's sarcoma. Amongst our patients three disorders stood out as distinctive early warning signs: chronic acneiform folliculitis; a florid beard and neck impetigo affecting the whole of the beard and front of the neck; and a fungus infection that was unusual in its severity and the number of sites involved. Although folliculitis, impetigo and fungal infections are commonplace, we would emphasize that the forms seen in our pre-AIDS and AIDS patients were of an extent or severity that we had not previously encountered.

To assess the significance of our observations, and the prevalence of other skin complaints in AIDS, we reviewed asymptomatic homosexuals and patients with AIDS and PGL as well as four high risk patients. When we came to attempt comparisons it was disconcerting to find that 32% of our apparently well homosexuals had HTLV-III antibodies. It is not possible to say how many of them, or of the seronegative controls, are incubating AIDS. Neither group can be considered entirely satisfactory as controls. However, if the diseased homosexuals, i.e. those with AIDS or PGL, are compared with seronegative homosexual controls in whom there is as yet no evidence of HTLV-III infection, a wide range of skin diseases is found in the AIDS and

TABLE 2. Prevalence of HTLV-III antibodies in AIDS, PGL and high risk patients and controls

| | No. of patients tested | No. of patients HTLV-III positive |
|---|---|---|
| AIDS | 9 | 9 |
| PGL | 15 | 13 |
| High risk | 2 | 2 |
| Controls | 144 | 46 |

TABLE 3. Occurrence of seborrhoeic dermatitis in AIDS and PGL patients and controls

| | No. of patients | | |
|---|---|---|---|
| | No rash | Seborrhoeic dermatitis | $P$* |
| AIDS | 8 | 1 | NS |
| Controls HTLV-III negative | 95 | 3 | |
| PGL | 11 | 4 | 0·003 |
| Controls HTLV-III negative | 95 | 3 | |
| Controls HTLV-III positive | 43 | 4 | NS |
| Controls HTLV-III negative | 95 | 3 | |

* Chi squared test incorporating Yates' correction. NS = not significant ($P > 0·1$).

PGL groups but not in the controls. Seborrhoeic dermatitis, mucocutaneous candidiasis and minor degrees of folliculitis, were found both in the control and in the AIDS and PGL groups.

Seborrhoeic dermatitis is common, but in our study we found it significantly more often in the PGL patients than in the HTLV-III negative homosexual controls, while there was no significant difference in the prevalence of seborrhoeic dermatitis between the AIDS patients and the controls (Table 3). Eisenstat and Wormser (1984) reported seborrhoeic dermatitis and a butterfly rash in AIDS, and Valle *et al* (1985) observed seborrhoeic dermatitis in men with the lymphadenopathy syndrome and with pre-AIDS (AIDS-related complex), but not in symptom-free HTLV-III positive men. Farthing *et al* (1985) also noted the occurrence of seborrhoeic dermatitis. They ventured to suggest that otherwise well HTLV-III positive individuals also had a higher prevalence of seborrhoeic dermatitis (20%). We found no significant difference in the prevalence of seborrhoeic dermatitis between HTLV-III positive and negative control subjects (Table 3).

The predisposition to infections in AIDS can be attributed to impaired immune responses. It has been suggested that the yeast *Pityrosporum ovale* is implicated in the pathogenesis of seborrhoeic dermatitis (McGinley *et al.*, 1975; Ford *et al.*, 1984), and it seems reasonable to suppose that the increased prevalence of seborrhoeic dermatitis in AIDS-related syndromes could be due to the overgrowth of this yeast.

The incidence of AIDS is inevitably going to increase. It should be noted that many patients will present with what at first appear to be trivial and innocuous skin complaints. Distinctive amongst these are a chronic acneiform folliculitis, a florid impetigo affecting the beard and neck and a widespread, severe fungus infection. We now view with some alarm the development of these disorders in homosexuals and other patients at risk.

## ACKNOWLEDGMENTS

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# The *in vitro* properties of dermal papilla cell lines established from human hair follicles

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## SUMMARY

The *in vitro* properties of cells cultured from the dermal papilla of human hair follicles were studied and compared with those of lines of dermal fibroblasts derived from the same material. In serial subcultures, the dermal papilla cells displayed a spread out, polygonal cellular morphology at stationary growth phases and a tendency to form multi-layered aggregates before reaching confluence. Aggregation was particularly marked when papilla cells were grown on collagen gels. In contrast, dermal fibroblasts grew as branching, parallel arrays of spindle-shaped cells which remained as monolayers until confluence. Compared with dermal fibroblasts, papilla cells also exhibited a shorter *in vitro* survival time. The properties of cultured human papilla cells are similar to those of rat vibrissa papilla cells.

The dermal component of the hair follicle is believed to play a fundamental role in the induction and maintenance of epithelial differentiation. In mammalian species, this has been studied most thoroughly in the rat vibrissa follicle, a model introduced by Cohen (1961). Oliver (1966a, b) demonstrated that whisker growth ceased when the dermal papilla or up to one third of the lower follicle was removed, and resumed when a new papilla formed from the fibrous root sheath. If more than one third of the lower follicle was excised, a new papilla was not formed and a hair did not grow. However, the implantation of an isolated dermal papilla into the lower end of such a cut follicle resulted in the generation of a hair bulb matrix from the outer root sheath and renewed hair growth (Oliver, 1967). It has also been shown that isolated vibrissa dermal papillae are able to induce the formation of follicular structures when associated with epidermis from glabrous ear or scrotal skin (Oliver, 1970).

In 1981, cell cultures were successfully established from the dermal papillae of rat vibrissa follicles (Jahoda & Oliver, 1981). Subsequently, it was demonstrated that cultured rat papilla cells exhibited unique properties which distinguished them from dermal fibroblasts (Jahoda & Oliver, 1984). These included a characteristic flattened morphology, a low proliferative activity and the formation of multi-layered aggregates at cell confluence. A method for culturing cells from human dermal papillae has recently been described (Messenger, 1984). In this paper we

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describe the *in vitro* properties of six human dermal papilla cell lines and compare them with those of six dermal fibroblast lines established from the same material.

## METHODS

### *Cell culture*

The method for establishing human dermal papilla cell cultures has been previously described (Messenger, 1984). Anagen follicles were dissected from biopsies of normal scalp tissue obtained during the excision of benign tumours. The hair bulb was excised and the fibrous sheath with attached dermal papilla was separated from the bulb epithelium by applying gentle pressure with the tip of a 21G needle. After the fibrous sheath was opened, the dermal papilla was transected across its stalk and transferred to the culture vessel. The isolated papillae were allowed to float freely in the culture medium in 25 cm$^2$ flasks. Dermal fibroblast cultures were also established from each biopsy. Hair follicles were removed as far as possible, the papillary dermis was cut into 1 mm cubes and placed in the culture flask. The culture medium used was TC 199 supplemented with glutamine, antibiotics and 20% foetal bovine serum. When sufficient cells were present in the primary culture they were harvested with Trypsin-EDTA and passaged as a single-cell suspension. Subsequent subcultures were carried out in a 1:3 split on confluent cultures. Some papilla cell lines would not grow to confluence, and these were passaged when the culture reached a plateau growth phase. To test the influence of the substrate on cell behaviour, third subculture cells were seeded onto collagen gels (Vitrogen). All cell culture materials were purchased from Flow Laboratories.

### *Growth kinetics*

Growth kinetic studies were performed on parallel dermal papilla and dermal fibroblast cultures at the fourth passage. $5 \times 10^4$ cells were seeded into 30 mm plastic petri dishes on day 0. Cells were harvested from duplicate dishes on days 1, 2, 3, 4, 5, 7, 10 and 14 and counted in a haemocytometer chamber. Three dermal papilla cell lines and three parallel dermal fibroblast lines were studied.

### *Immunofluorescence*

To exclude the possibility that cells cultured from the papilla explants were of epithelial or endothelial origin, the cultures were stained for cytokeratin and factor VIII-related antigen. A broad-spectrum monoclonal cytokeratin antibody (Labsystems), a polyclonal keratin antibody (Dako) and a polyclonal Factor VIII-RA antibody (Dako) were used. Cells were grown on glass slides for 2 days, fixed in cold methanol and washed thoroughly with buffer. The cultures were incubated with the primary antibody for 15 min, washed, and then incubated with the appropriate FITC-conjugated second antibody for a further 15 min. After washing in buffer, the slides were mounted with coverslips and viewed in a fluorescence microscope. Positive controls for cytokeratin were provided by cultures of epidermal keratinocytes and for Factor VIII-RA by umbilical vein endothelial cell cultures.

## RESULTS

The initial outgrowth of cells from the dermal papilla explants occurred after about 5 days. These cells displayed a flattened, polygonal morphology with several processes of varying lengths extending from the edges of a striated cytoplasm. Proliferation in the primary culture

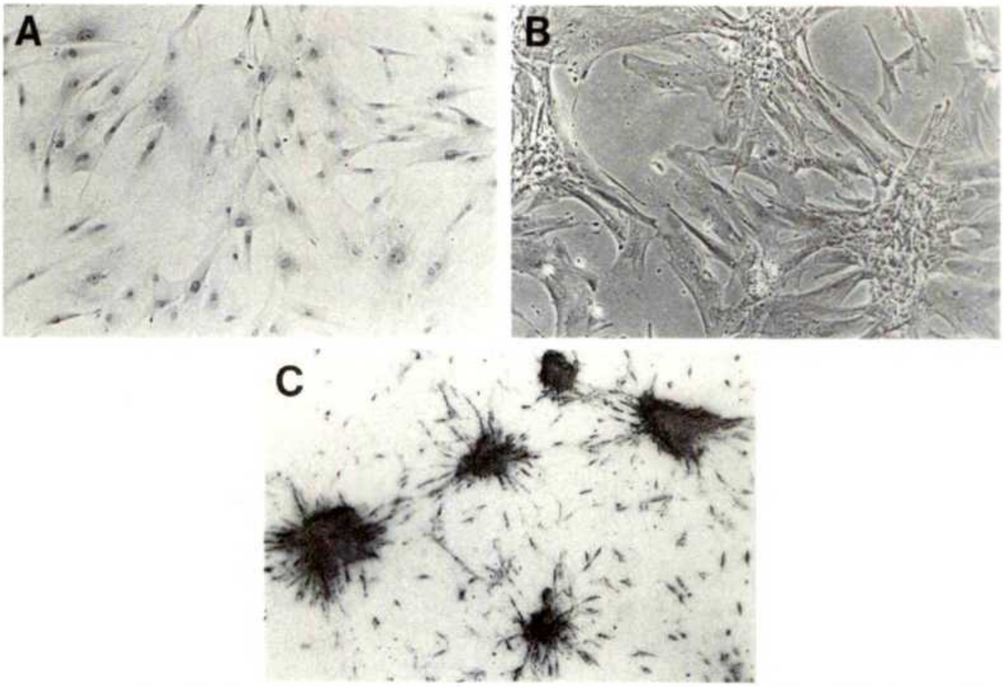

FIGURE 1. Dermal papilla cells; (A) isolated papilla cells show a flattened spread-out morphology and a striated cytoplasm ( × 100); (B) as their number increases, papilla cells form into multi-layered aggregates separated by areas of uncovered substratum. Cells around the edges of aggregates show a spread-out morphology ( × 100); (C) marked clumping of papilla cells seeded onto collagen gel. Photograph taken 24 h after seeding ( × 40).

was heralded by the appearance of more spindle-shaped cells which formed into multi-layered parallel arrays and spread slowly across the substratum. This occurred after 3–4 weeks. Papilla cells subcultured at low density exhibited the same flattened morphology seen during the migratory phase in the primary culture (Fig. 1a). As their numbers increased, the cells associated into aggregates and clumps which became multi-layered before confluence was reached in all six lines (Fig. 1b). This aggregative behaviour was more pronounced in later subcultures after five passages. Between adjacent aggregates, areas of uncovered substratum were often apparent even when a stationary growth phase had been reached. In early subcultures the degree of aggregation was variable between different cell lines. Four papilla lines initially showed a more typical fibroblastoid growth pattern, forming branching, parallel arrays of spindle-shaped cells, although multi-layering before confluence was a consistent feature. Two papilla lines displayed marked aggregative behaviour, and failed to grow to confluence throughout their life span.

Dermal fibroblasts mainly showed a bipolar, spindle-shaped morphology (Fig. 2a) which was consistent in any single cell line, although some variation in morphology was noted between different lines. At low cell densities, fibroblasts often displayed a broadened, ruffled membrane at one pole. With increasing cell numbers, dermal fibroblasts formed into branching, parallel arrays (Fig. 2b). They exhibited little or no multi-layering, remaining as monolayers until confluence was reached.

When papilla cells were seeded onto collagen gels their aggregative behaviour was greatly

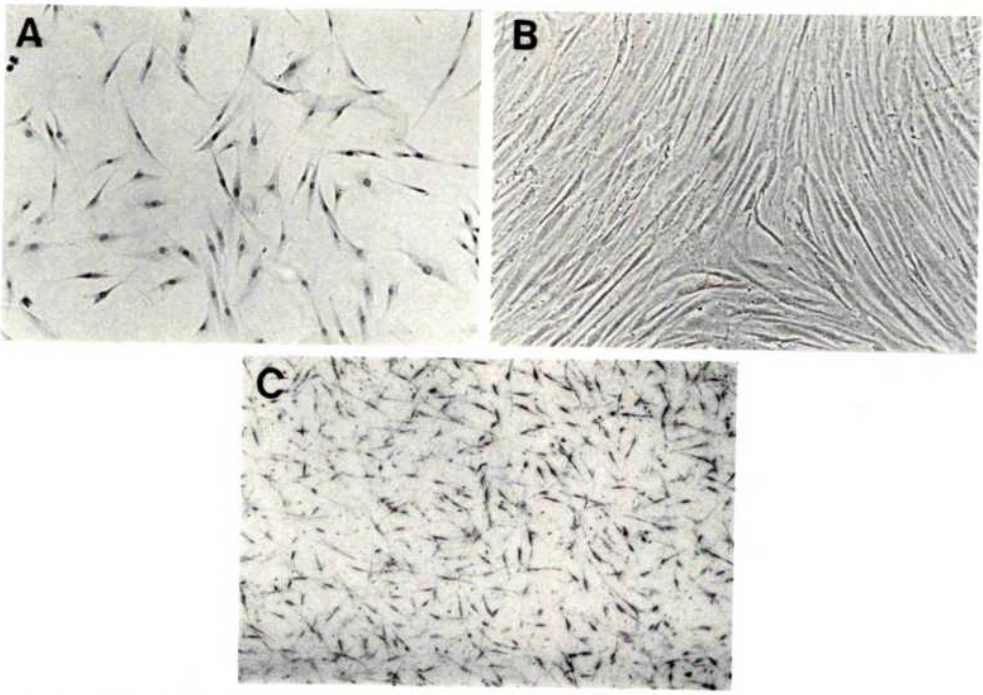

FIGURE 2. Dermal fibroblasts; (A) isolated cells show a more bipolar, spindle-shaped morphology (× 100); (B) cells form into branching parallel arrays as their number increases (× 100); (C) cells remain distributed uniformly over the substrate when seeded onto collagen gels (× 40).

enhanced. Within 24 h, large, dense clumps of cells had formed over the gel surface (Fig. 1c). Cells around the edges of these clumps were arranged with their long axes directed towards the centre. One dermal fibroblast line also showed a clumping tendency on collagen gel although this was not as marked as in the papilla cells. In the other five lines the cells remained uniformly distributed on the gel surface (Fig. 2c).

*Growth kinetics*

Mean growth curves of three papilla cell lines and three concurrently established dermal fibroblast lines are shown in Figure 3. During the log phase, the mean population doubling times of dermal fibroblasts and dermal papilla cells were similar (59·2 h and 66·9 h respectively), although maximum proliferative activity occurred earlier in dermal fibroblasts at days 2–5 by comparison with papilla cells at days 4–7. Final cell numbers were greater in dermal fibroblast cultures than in papilla cell cultures but the difference was not statistically significant. After about five subcultures the growth rates of papilla cells declined and this was reflected in an increasing interval between passaging. Senescence was deemed to have taken place when a single population doubling failed to occur within 14 days. This stage was reached after 5–10 subcultures in the six papilla cell lines. One dermal fibroblast line became senescent after five subcultures. Of the other five dermal fibroblast lines, three were passaged beyond 15 subcultures and two beyond 20 subcultures.

Immunofluorescence staining of dermal papilla cultures for cytokeratin and Factor VIII-RA was negative.

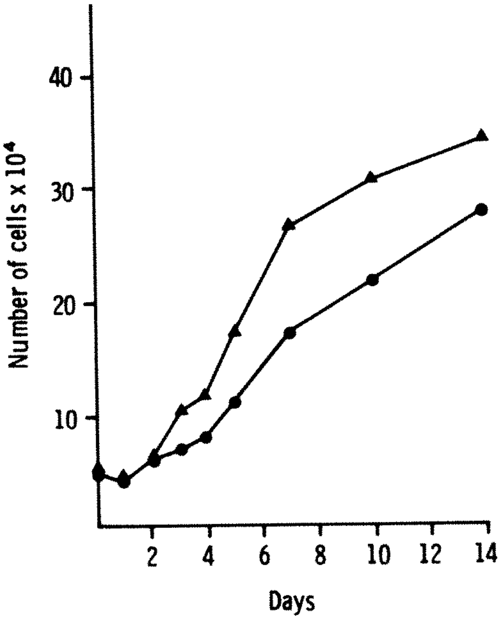

FIGURE 3. Mean growth curves of three lines of dermal papilla cells (●) and three simultaneously established lines of dermal fibroblasts (▲).

## DISCUSSION

Heterogeneity in fibroblast-like cell lines is well recognized. Conrad, Hart and Chen (1977) described differences in fibroblast morphology and social behaviour in cell lines grown from three separate body sites. Differences in proliferative activity between fibroblasts grown from the papillary and reticular dermis of human skin have also been reported (Harper & Grove, 1979; Azzarone & Macieira-Coelho, 1982). Human dermal papilla cells display morphological and behaviour properties which are sufficiently distinctive to suggest that they represent a separate population of skin fibroblasts. These characteristics are similar to those seen in rat papilla cultures (Jahoda & Oliver 1984) although the difference in proliferative activity between papilla cells and dermal fibroblasts was not as marked.

The dermal papilla arises as a condensation of mesenchymal cells early in the course of hair follicle morphogenesis and therafter remains as a discrete structure throughout subsequent hair cycles. Although from an early stage in development papilla cells exhibit little or no proliferative activity (Wessells & Roessner, 1965; Pierard & de la Brassinne, 1975), profound changes occur in cellular morphology, cell contacts and synthesis of extra-cellular matrix during the hair cycle (Montagna *et al.*, 1952; Young, 1980). The early development of the lower part of the adult anagen follicle, a process in which the dermal papilla probably plays an important inductive role, resembles embryonic follicle development. Similarities have also been described between the ultrastructure of the adult and developing dermal papilla (Hashimoto, 1970; Hashimoto & Shibazaki, 1976). The aggregative properties displayed by both rat and human dermal papilla cells are an unusual and prominent feature of their behaviour. The affinity of these cells for one another is also evident from the observations that isolated dermal papillae maintain their integrity for many months when implanted into the dermis (Jahoda & Oliver, 1984). These workers suggested that cell aggregation in papilla cultures may be analogous to mesenchymal condensation and may thus provide an adult model for studying this vital stage in follicle development.

A major limitation of cell culture systems is the difficulty of establishing whether the cells retain their *in situ* function. It has now been shown clearly that rat papilla cells, in early subcultures, possess the ability to induce hair growth in the vibrissa follicle (Jahoda, Horne & Oliver, 1984). The ability to culture large numbers of cells from human dermal papillae (up to $10^9$ from a single papilla) may allow similar studies to be carried out in man, although different experimental techniques will be necessary. It will be possible also to study a number of other aspects of papilla cell function such as the nature of their aggregative behaviour, their biosynthetic activities (for collagen and glycosaminoglycans), and alterations which may occur in disorders of hair growth.

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# Histochemical and morphological studies on mammalian epidermal peridermal granules

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## SUMMARY

We performed morphological studies on the epidermal peridermal granules (PG) of newborn mice using histological, fluorescent and electron microscopic methods. PG appeared during the 17th to 18th day of foetal life, and remained in the outermost epidermal layers until 5 days after birth. PG were evenly distributed over the entire cutaneous surface of the mouse, except the tail. PG were found to contain protein and corresponded to sites of transglutaminase activity, but they were devoid of lipids and nucleic acids. They could be differentiated clearly from keratohyalin granules. Together, these studies suggest that PG represent a unique epidermal protein product that serves as a marker of late foetal development.

Peridermal granules (PG) have been noted by several investigators in studies on epidermal embryogenesis (Bonneville, 1968; Stern, 1970; Stern, Dayton & Duecy, 1971; Holbrook & Odland, 1975; Buxman, Kocarnik & Holbrook, 1979). In the mouse, these small granules (0·4–4 μm in diameter) appear just below the peridermal layer on the 17th to 18th day of foetal life, and persist up to, and shortly after, birth (Bonneville, 1968). The appearance of these distinctive beaded structures along, or above, the outer layer of the stratum corneum during embryogenesis has prompted speculation about their function and their possible correlation with epidermal differentiation (Stern *et al.*, 1971; Bonneville, 1968). However, previous efforts to determine the composition of these granules have yielded little information (Stern, 1970).

In the present study we have attempted to characterize PG further, by (1) determining their composition histochemically, and (2) delineating the time course of their appearance and disappearance in foetal and post-natal skin. We used a new method for the detection of PG employing the fluorescent dye, 8-anilino-1-naphthalene sulphonic acid (ANS).

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## METHODS

The following studies were performed to characterize PG in the skin of foetal and newborn mice: (1) distribution of the granules over the head and neck, ear, back, abdomen and chest, side and shoulder, flank, and tail; (2) determination of the depth within the stratum corneum that PG could be detected; (3) length of time after birth for which PG persist, (4) analysis of PG composition using lipid solvents, histochemical stains, immunocytochemistry and enzyme digestion.

### *Preparation of epidermis*

Newborn and pregnant ICR mice were obtained from Simonsen Laboratories, Gilroy, CA, U.S.A. Animals were sacrificed by cervical dislocation and biopsy specimens of back skin were taken daily from foetal day 18, and from newborn mice from the day of birth to post-natal day 5. Except for routine histological and electron microscopic studies (see below), experiments were performed on unfixed frozen sections (6–8 μm thick), cut on a Universal cryostat. Frozen tissue sections were stored at −70°C, and, except where noted, tissues were examined within one week.

### *Electron microscopy*

Samples for ultrastructural analysis were fixed in 1·5% glutaraldehyde in 0·1 M cacodylate buffer, containing 4% sucrose and 0·5% $CaCl_2$, pH 7·4, for 4 h at room temperature, washed three times in 0·1 M cacodylate buffer containing 7% sucrose, post-fixed in 1% aqueous osmium tetroxide, pH 7·2, stained *en bloc* with uranyl acetate, dehydrated in graded ethanols, and embedded in Epon. Thin sections were examined either unstained or double-stained with lead citrate and uranyl acetate in a Zeiss 10A electron microscope at 60 kv.

### *Tape-stripping studies*

Newborn mice were tape-stripped with double-stick cellophane tape (Scotch®, Minnesota Mining and Manufacturing #665 double-coated tape). One side of the tape was placed on a clean glass slide and the open face pressed firmly against the outer surface of the skin. The tape was handled with forceps so that tape-stripped stratum corneum cells alone adhered to the tape. Fluorescent dye markers were applied drop-wise to the cells attached to the tape, and slides were incubated for 30 s to 3 min at 25°C. The slides were rinsed briefly in distilled water and cover-slipped in distilled water or Aqua-mount (Sigma, St. Louis, U.S.A.), and both the numbers and sizes of PG were estimated subjectively in a Leitz Ortholux II light microscope equipped for epifluorescence.

### *Solvent extraction*

The following solvents were employed: absolute acetone, chloroform, ethyl alcohol, phosphate-free diethyl ether, methanol and tetrahydrofuran (THF) (all reagent-grade). The effect of the detergents, SDS and Triton X-100, was also determined. The solvent was applied to the skin with a cotton-tipped applicator. After letting the skin dry for 1–5 min, the treated side and the untreated contralateral side (control) were tape-stripped, and cornified cells examined for the presence of PG with fluorescent dyes.

### *Fluorescent techniques and identification of transglutaminase*

Several fluorescent tracers were tested for labelling of PG in tape stripped cells and in frozen sections. These were 8-anilino-1-naphthalene sulphonic acid (ANS) (specific for proteins and lipids), acridine orange (acidic polysaccharides, free and bound RNA and DNA), filipin (cholesterol), and fluorescein isothiocyanate (FITC) (free and bound proteins). Of these, only ANS stained PG distinctly, although PG were also stained by aqueous 1% acridine orange (Abrams & Elias, 1962; Wiatr, 1976) between pH 7 and 9 (Table). However, PG were not visualized when acridine orange was applied to fresh tissues, appearing only after tissue had been frozen and stored for over a week. Since ANS fluorescence is partially quenched in water, and its fluorescence is augmented in the presence of ethanol (Slavik, 1981), we used a concentration of $10^{-2}$ M to $10^{-3}$ M ANS in 80% ethanol. The use of a lower concentration of ANS ($10^{-3}$ M), together with a briefer application (less than 1 min), allowed PG to be distinctly stained in frozen sections of the stratum corneum. Fluorescence microscopy was performed with a Leitz Vario-Orthomat microscope using a BG-10 filter for both ANS and acridine orange (450 nm wavelength excitation).

Identification of transglutaminase in fresh-frozen sections was performed with monodansyl cadaverine (Sigma) by the method of Buxman and Wuepper (1978).

### *Immunofluorescence*

Foetal mice (> 17 days) were flash frozen in cryostat freezing medium. Sections of approximately 7 μm were cut on a cryostat, washed in PBS, fixed in absolute methanol for 5 min, washed, and pre-incubated in normal goat serum for 30 min (this step was necessary to reduce non-specific binding of fluorescein-conjugated antiserum). Sections were incubated for 30 min with antibody to rat filaggrin using dilutions of 1/100 to 1/1000 or with equivalent dilutions of pre-immune serum or antibody absorbed with purified rat filaggrin (Olmsted, 1981), washed in two changes of PBS, then incubated with goat anti-rabbit IgE (Capell Laboratories, Cochranville,

TABLE. Summary of histochemical, extraction, and digestion studies on peridermal granules (PG)

| Affects PG (specificity) | No effect on PG (specificity) |
|---|---|
| Stains: | |
| Haematoxylin (basic proteins ribonucleoprotein) | Oil red o, fat red 7B (neutral lipids) |
| Monodansyl cadaverine (transglutaminease) | Nile blue sulphate (acidic lipids, free sterols) |
| Coomassie Blue (proteins) | Osmium tetroxide (unsaturated lipids) |
| | Ninhydrin-Schiff (amino groups) |
| | Periodic acid-Schiff (carbohydrates, glycoconjugates) |
| | Chlortetracycline (calcium) |
| Solvents/detergents* | |
| Chloroform** | Acetone |
| Diethyl ether** | Ethanol |
| Sodium dodecyl sulphate | Tetrahydrofuran |
| Digestion* | |
| None | 1·0 M phosphate buffer, pH 7·0 |
| | 8·0 M urea, pH 8·4 |
| | 1 μg/ml RNAase |
| | 10 μg/ml protease K |
| | 0·5% trypsin |

* Peridermal granules were visualized with $10^{-3}$ M ANS in 80% ethanol (*see* Methods).

** Granules were not dissolved but were released intact into the supernatant.

PA, U.S.A.) for 30 min. Slides were washed again, and coverslipped with 90% glycerol/0·1 M Tris-HCl, pH 9·6, and observed on a Zeiss microscope equipped with epifluorescence.

### *Digestion, extraction, and histochemical studies*

Digestion experiments were performed both on whole sheets of stratum corneum, obtained with staphylococcal epidermolytic toxin as previously described (Elias *et al.*, 1979), and on fresh-frozen sections, placed on gelatin-coated glass slides. Whole newborn mouse stratum corneum was immersed either in 0·5% trypsin (Sigma) in pH 7·4, for 1 h at 37°C, or in 1·0 M potassium phosphate buffer, pH 7·0, for 30 min (Ugel, 1969; 1971), or in 8 M urea in tris-buffered saline (TBS), pH 8·4, for 1 h (Ball, Walker & Bernstein, 1978). Treatment of frozen sections (8 μm thick) at 37°C was performed with either 8 M urea, or 1 M potassium phosphate buffer (as above) or with 10 μg/ml protease K (Sigma) in TBS, pH 7·4, for 10 min, or 1 mg/ml RNAase (Sigma) in 0·005 M phosphate buffer, pH 7, for 0·5 h (Ugel, 1971). After digestion, the sections of tissue were stained with $2 \times 10^{-3}$ M ANS in 80% ethanol, and observed under the fluorescence microscope.

A battery of routine histochemical stains (Pearse, 1982; Bancroft & Stevens, 1982) was applied to fresh-frozen sections (6 μm), before and after enzyme digestion or solvent extraction (Table). Stained sections were then examined by transmitted light in the Leitz microscope.

## RESULTS

PG appear to be quite inert or 'durable', since they could be seen clearly in tape-stripped samples even after 3 months, requiring only a re-application of ANS solution. PG also could be visualized in tissue specimens frozen for 2 months, even when the remainder of the tissue had undergone considerable deterioration.

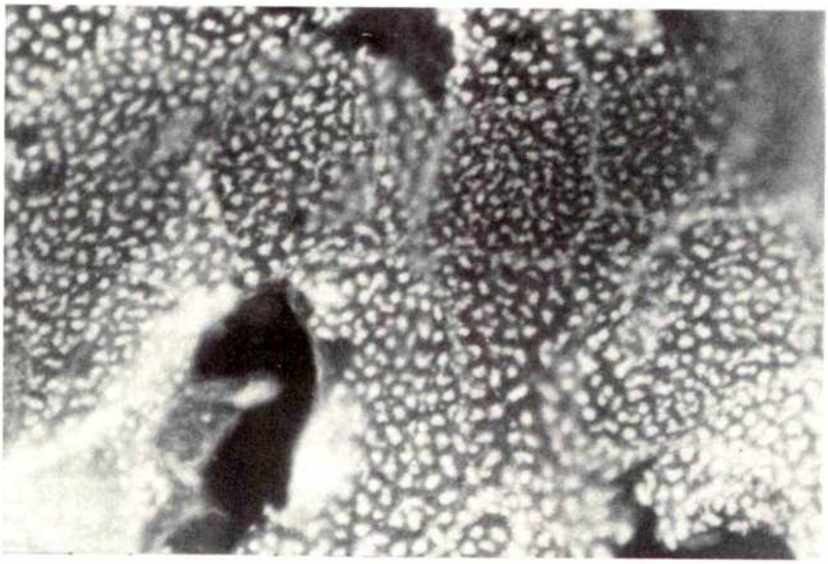


FIGURE 1. Tape-stripped cells from a 1-day-old mouse, stained with $10^{-2}$ M ANS in distilled water. (Original × 125).

### *Tape-stripping studies*

PG were detected in foetal mice from day 17 and in newborn mice from the day of birth until the fourth post-natal day (Fig. 1). Occasionally, a few patches of PG could be seen in 5-day old mice. As the mice grew older (up to 5 days), the PG became coarser and larger in size, as well as decreasing in number in the first tape-stripped layer (Fig. 2). Generally, PG were evenly distributed over any particular region (Fig. 1), with the distribution becoming more patchy and irregular as the mice grew older. However, tape-stripping of the tail revealed no PG, and little evidence of PG was found in the head and neck regions. Tape-stripped stratum corneum from hairless mice, adult ICR mice (ears and tail), and adult human skin did not reveal any PG.

In the five tape-stripped layers from one-day-old mice, PG were found almost exclusively in the first and second layers, with more PG in the first layer than in the second. A small number could be detected occasionally in the third, and rarely in the fourth tape-stripped layers, but these probably reflected residual cells that did not detach with stripping of the more superficial layers. Examination of the stratum corneum of mice between 2 and 5 days of age revealed a steady diminution of PG with time, until only one or two cornified cells contained PG in a sheet of otherwise negative cornified cells.

Examination of cross-sections of the stratum corneum by light and fluorescent microscopy suggested that PG protruded from the apical surfaces of cornified cells (Fig. 3). However, on electron microscopy, PG were found to be located in the cornified cell cytoplasm, causing prominent, focal protrusions of the apex of the cornified cell envelopes (Figs 4 & 5). In contrast to intercellular lipids in mammalian stratum corneum, which are extracted during processing and embedding (Elias, Goerke & Friend, 1977), these structures were electron-dense and resisted the usual extraction of intercellular lipids that occurs during processing of tissue samples for electron microscopy.

The most effective single agent for removal of PG was diethyl ether, followed by chloroform, but SDS and Triton were also effective. None of the other treatments substantially affected the

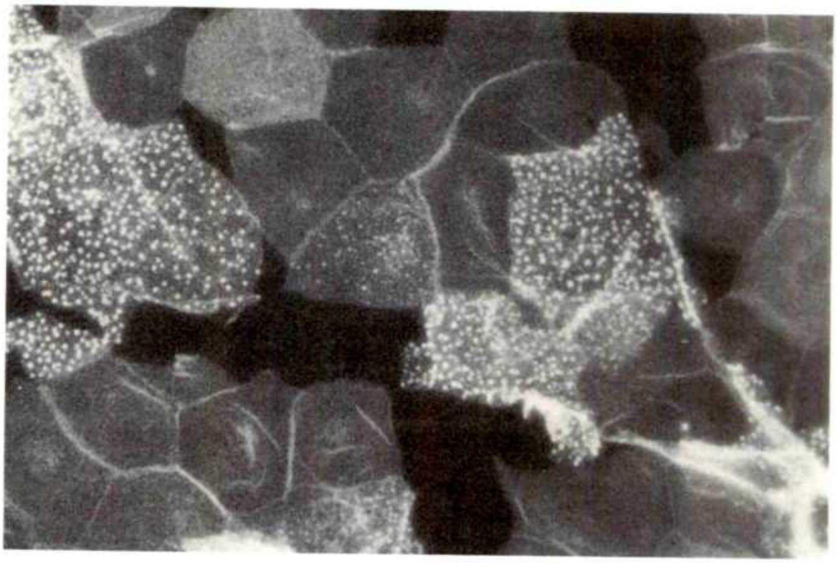


FIGURE 2. Tape-stripped cells from a 3-day-old mouse. At this age, PG are found on some, but not on all of the cornified cells stained with $10^{-2}$ M ANS in distilled water. (Original × 125).

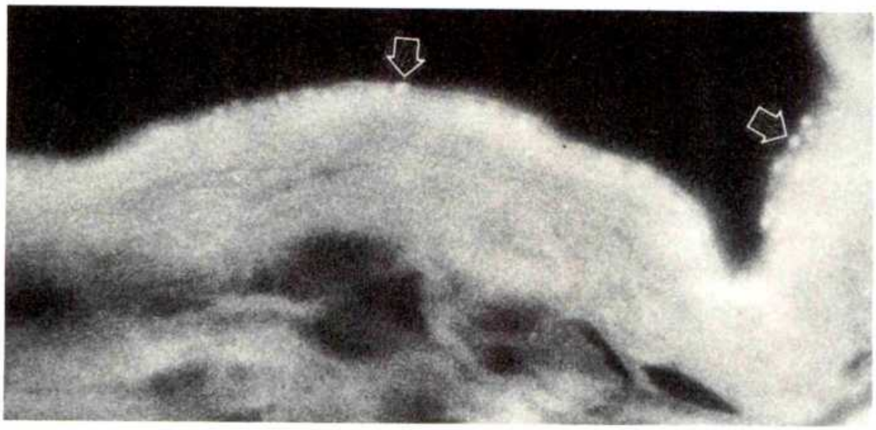
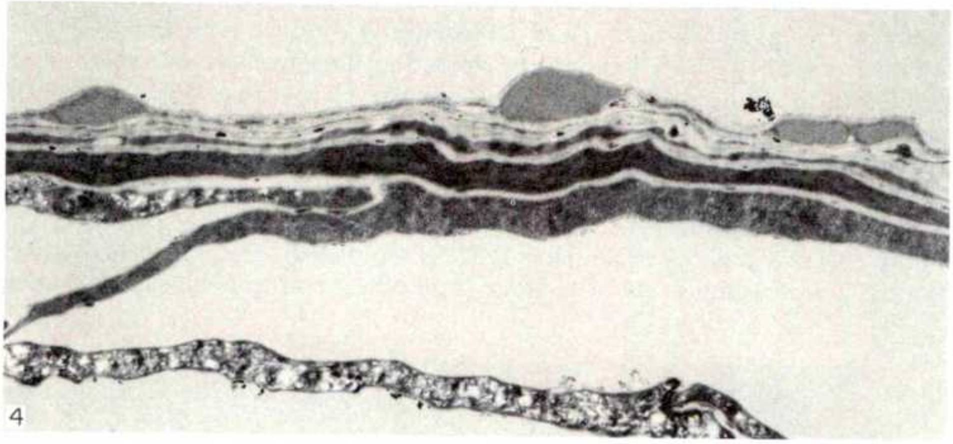
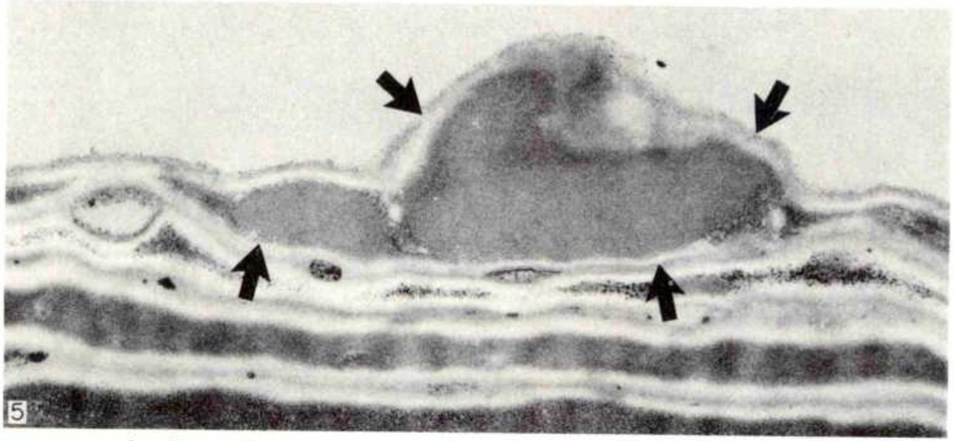


FIGURE 3. Frozen section (8 μm) of the skin of a 1-day-old mouse. PG appear along the most superficial layer of the stratum corneum. Stained with $10^{-3}$ M ANS in 80% ethanol. (Original × 750).





FIGURES 4 and 5. Low and high magnification electron micrographs of PG on the skin of a newborn mouse. The PG are enclosed by a thickened stratum corneum envelope (arrows, Fig. 5). (Fig. 4, original × 12500); (Fig. 5, original × 40000).

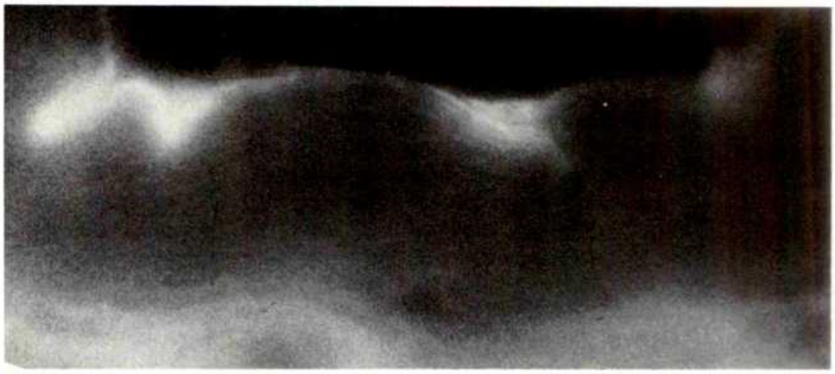


FIGURE 6. Frozen section of the skin of a newborn mouse stained with monodansyl cadaverine (MDC), indicating the presence of transglutaminase activity. In some areas where the stratum corneum is thicker, MDC staining can be discerned as distinct spots. PG were localized in the same areas on an accompanying section stained with $10^{-3}$ ANS in 80% ethanol. (Original × 125).

PG. When the skin of 2-day-old mice, and tape-stripped cells, were washed in a small amount of diethyl ether or chloroform, small aggregates of brightly fluorescent bodies were seen in the supernatant, suggesting that these two solvents may remove PG intact from the stratum corneum, rather than dissolving them.

### *Histochemical characterization (Table)*

In unfixed, unstained frozen sections of 1 or 2-day old mice, PG could be seen easily as round protrusions of the outer stratum corneum. Superimposition of fluorescent and light microscopic images demonstrated the coincidence of both the ANS-stained bodies and the unstained round bulges.

Although PG were dissolved by both diethyl ether and chloroform, they were not stained by any of the lipid stains employed, including nile blue sulphate, which has been noted previously to stain maturing foetal skin (Brosens, 1966; Sharp, 1968; Chan, Willis & Woods, 1969). Despite earlier suggestions that the periderm, and PG in particular, may function in either glucose uptake from the amniotic fluid or in glycogen modification, PG were not stained with PAS with or without diastase.

Since ANS stains both lipids and proteins, the fluorescent tracer was further modified to see if PG might contain protein. Since proteins demonstrate a higher affinity for ANS than do lipids (Slavik, 1982), we reduced the concentration of ANS to $10^{-3}$ M and shortened the staining times (to less than 1 min), to preferentially stain proteins. This modification revealed limited fluorescence at the border of the stratum corneum, where PG were visible as bright green beads (Fig. 3), and the lipid-rich intercellular domains were unstained (Elias *et al.*, 1977; 1981). The protein nature of PG was supported both by the strong blue staining with Coomassie Blue and faint blue staining with Harris' haematoxylin. However, PG were not stained with ninhydrin-Schiff stain for free amino groups.

### *Enzyme localization, immunocytochemical and digestion studies*

Since PG in foetal epidermis appear concurrently with the emergence of the enzyme transglutaminase, a later differentiation marker in the foetal epidermis (Buxman *et al.*, 1979), we examined whether PG might be the site of transglutaminase activity in the epidermis. Whereas

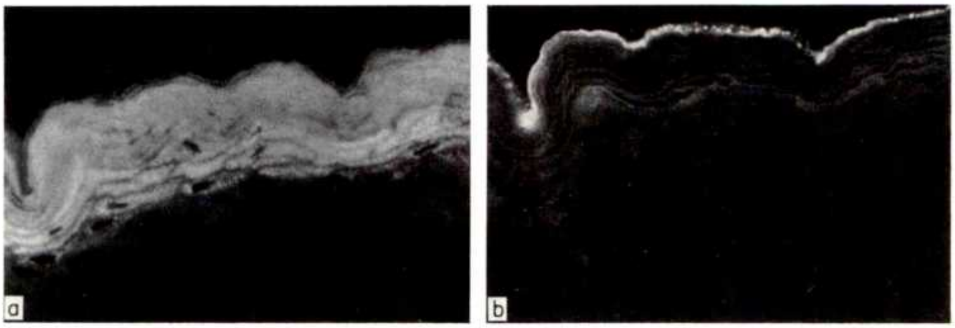


FIGURE 7. Immunofluorescence of 18-day-foetal mouse skin using antibody to rat filaggrin. (a) Anti-filaggrin serum. Note strong positive reaction of keratohyalin granules and stratum corneum. Periderm granules are also positive; (b) pre-immune normal rabbit serum. Note that the peridermal granules are positive in this control indicating that the reaction with antifilaggrin serum above is non-specific. A similar result was obtained using antiserum absorbed with filaggrin. (Original × 200).

there was a bright band of fluorescence located in the regions of the outermost stratum corneum where PG occur (Fig. 6; PG were detected by ANS fluorescence on a corresponding tissue section), it was not possible to verify unequivocally that transglutaminase activity was localized to PG.

Since both the staining properties and the ultrastructural appearance of PG are consistent with a relationship to keratohyalin granules (Bauer, 1972; Bonneville, 1968; Stern *et al.*, 1971), we performed digestion and extraction studies to determine whether PG exhibited properties of keratohyalin granules (KHG). However, both 1 M phosphate buffer (histidine-rich protein; Ugel, 1969; 1971) and 8 M urea (stratum corneum basic protein, Dale & Ling, 1979; Dale, 1977; Ball *et al.*, 1978) failed to remove PG. Moreover, in frozen sections labelled with rabbit anti-filaggrin antibody, no specific binding of antibody to PG was apparent on indirect immunofluorescence (Fig. 7). Trypsin, protease and RNAase-treatment also left PG intact. Finally, although calcium has been implicated in the control of epidermal differentiation, fluorescent staining of PG was not obtained with chlortetracycline. Thus, PG do not appear to be related chemically to either the histidine-rich component of KHG or to stratum corneum basic protein.

## DISCUSSION

In this study we have demonstrated the rapid and relatively specific labelling of epidermal PG in both fresh-frozen thin sections and in fresh tape-stripped cells, with the fluorophore ANS, suspended either in 80% ethanol or in distilled water. PG first appear on day 17 or 18 in developing foetal mouse epidermis (*see also* Stern *et al.*, 1971). That PG disappear after birth suggests either that they have an *in utero* function, or that they are a vestigial differentiation marker of developing epidermis. Although the present studies were done with ICR mice, we also have seen PG in newborns of other mammalian species, including the CH3/AN mouse strain and Sprague-Dawley rats. Similar structures have been seen in embryonic chicken (Matoltsy, 1966; Elias & Friend, 1976), and human (Holbrook, 1979) skin.

We have evidence that PG are of a proteinaceous composition, because we found positive fluorescence staining with dilute ANS, faint-blue staining with haematoxylin, and strong staining with Coomassie Blue. Stern *et al.* (1971) and Bonneville (1968) also were able to stain

PG in Epon-embedded sections, utilizing Richardson's stain and toluidine blue, and faint staining with haematoxylin also was seen by Stern *et al.* (1971). These workers also noted that KHG stained differently from PG, with the latter displaying a darker, bluish-purple colour suggesting, as do our studies, that KHG and PG are not identical.

The resistance of PG to both trypsin and urea degradation is similar to the behaviour of the KHG granule component, isolated by Matoltsy (1962), which also was resistant to trypsin and urea treatments. Both Matoltsy (1962) and Bonneville (1968) suggested that PG may represent a 'nucleation region' or a complex of nucleoprotein and KHG, where typical KHG are formed. As PG were stained by both haematoxylin and acridine orange, they may have a nucleoprotein component. However, the failure of PG staining to be altered by RNAase and protease digestion would indicate that it is not a ribonucleoprotein.

The larger question still remains of whether PG represent structures derived from the periderm or from the epidermis. Bonneville (1968), Bauer (1978) and Ball *et al.* (1978) and found that various forms of KHG, generated during epidermal differentiation *in utero*, develop from the underlying epidermis rather than from the periderm. In contrast, Bonneville (1968) and Bauer (1972) noted that PG appear initially in either the inner peridermal layer or within the most superficial layer of the stratum corneum. Freinkel (quoted by Holbrook, 1983) suspected that this peridermal granule-containing layer may actually represent the outermost layer of the epidermis. It has been suggested frequently that PG may be a precursor or degradation product of KHG (see, for example, Bonneville, 1968). However, by both immunocytochemistry and histochemical techniques, we have shown that, while PG also appear to be protein and although they resemble KHG in electron micrographs, they are distinct from adult KHG. These findings suggest that PG are derived from the periderm, rather than the epidermis, and support the distinction between these two tissues.

The tails of new born mice were devoid of PG, and this difference may allow further studies to determine their nature. A correlation between PG appearance and transglutaminase activity suggests a possible function for this structure in terminal epidermal differentiation. Others have suggested that disappearance of PG somehow signals the beginning of cornification and desquamation (Bonneville, 1968; Stern *et al.*, 1971). Thus, the peridermal granules could facilitate cornification via release of transglutaminase, a calcium- and sulphydryl-dependent enzyme, to the underlying epidermis, with a role for this enzyme in the subsequent stablization of the keratinocyte cell membrane.

Although their numbers are too sparse to allow isolation and full characterization, further immunocytochemical studies with monoclonal antibodies to envelope precursor proteins, or to transglutaminase could help to distinguish between the various possible functions of PG.

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# Immunohistochemical staining of normal sweat glands

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## SUMMARY

Sweat glands from normal skin obtained at autopsy or as routine biopsies were examined using a panel of immunoperoxidase-linked antibodies. The results indicate that such a panel of antibodies defines all known functional regions of the eccrine sweat gland and provides a reliable distinction from all other skin elements.

The human eccrine sweat glands have been studied extensively and their anatomy and physiology have recently been reviewed (Hashimoto, 1978a). The apocrine sweat glands have received less attention but current knowledge regarding them has also been recently reviewed (Hashimoto, 1978b). The biochemistry and pharmacology of eccrine glands has been reviewed by Sato (1983) and that of apocrine glands by Robertshaw (1983). Studies of sweat glands using immunoperoxidase methods have been reported sporadically in the literature but as yet no systematic studies of normal sweat glands have been performed in order to provide a baseline for the investigation of pathological conditions. We have therefore studied normal human sweat glands using a large battery of immunoperoxidase markers, preparatory to studying the various sweat gland tumours of the skin.

## METHODS

Twenty skin specimens were examined; 14 from fresh routine surgical material or from skin samples previously received and coded as normal on the SNOP (Systematised Nomenclature of Pathology) retrieval system, and six from routine autopsy material within 12 h of death. The autopsy specimens were taken from two subjects at three sites: forearm, calf and sole. The skin specimens were processed in the routine manner through paraffin wax, and sections were cut at 5–7 $\mu$m and stained with haematoxylin and eosin. Appropriate specimens were selected from these and further paraffin sections were cut at 3–5 $\mu$m for immunoperoxidase staining.

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TABLE 1. Staining of normal sweat glands with antibodies

| | Portion of eccrine apparatus stained | | | | |
|---|---|---|---|---|---|
| Antibodies | Acro-syringium | Straight duct | Coiled duct | Secretory coil | Apocrine gland |
| 28 (hair-derived antibody) | − | +/− | − | − | − |
| 29 (callus) antibody | − | + | + | + | − |
| CAM 5·2 | − | − | − | + | + |
| HMFG1 | − | − | − | − | + |
| E29·68 | − | − | − | + | + |
| CEA | + | + | + | + | − |
| Carbonic anhydrase | − | − | − | − | − |
| Brush border | − | +/− | +/− | − | +/− |
| S-100 | − | − | − | +/− | − |

+ Positive reproducible staining;
+/− Non specific or unreproducible staining;
− No staining.

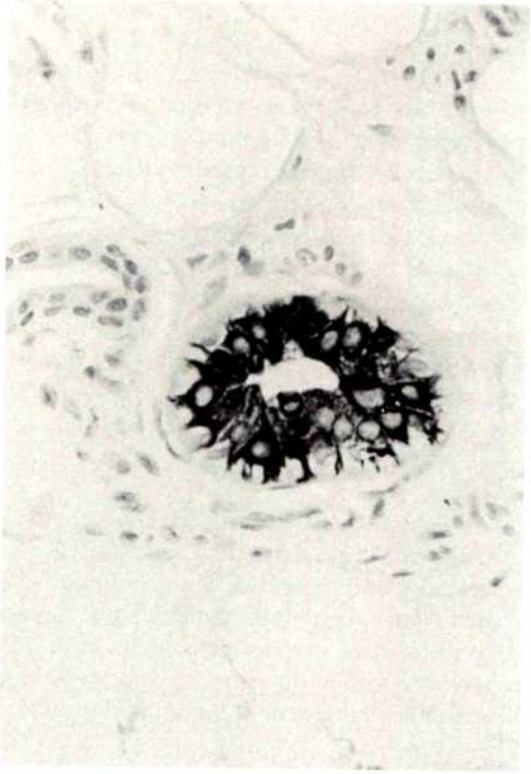

FIGURE 1. CAM 5·2 antibody staining of sweat gland secretory coil, the adjacent coiled duct is unstained.

### *Immunocytochemistry*

The sections were stained with polyclonal antibodies using a modified peroxidase-anti-peroxidase (PAP) technique with prior trypsinization (Mepham, Frater & Mitchell, 1979) and with monoclonal antibodies using an indirect immunoperoxidase technique. Anti-CEA, S-100, and PAP, swine anti-rabbit and rabbit anti-mouse immunoglobulins were obtained from Dako Immunologicals. HMFG1 was purchased from Seward Laboratories. E29.68 was a gift from Dr D.Y. Mason, Department of Haematology, John Radcliffe Hospital, Oxford, U.K. Both are milk fat globule antibodies. CAM 5·2 was a gift from Dr W. Bodmer ICRF, Lincolns Inn Fields, London, U.K. The keratin antibodies 28 and 29 were prepared as described by Cotton, Kirkham and Young (1984). 28 is raised against hair clippings with no hair bulb, and 29 to callus. Carbonic anhydrase antibody was prepared in the Department of Neuropathology, Southampton General Hospital, U.K. (Steart, 1983). Brush border antibody was prepared according to the method of Wallace *et al.* (1962).

## RESULTS

Table 1 lists the staining reactions seen in eccrine and apocrine sweat gland structures. Serum 28 (hair-derived antibody) stained the basal layer of the epidermis fairly strongly and the other layers in a weaker and more diffuse pattern, but it showed very specific staining of the hair root

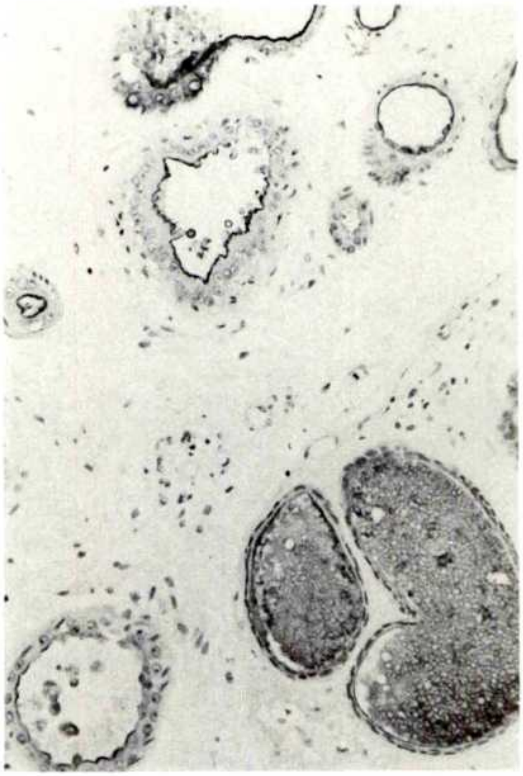

FIGURE 2. CAM 5·2 antibody staining of apocrine secretory coil and duct.

and the trichilemmal layer of the follicles. It showed no staining of sweat gland elements except for a very weak and diffuse reaction with the lumen of the straight duct.

Serum 29 (callus-derived antibody) reacted strongly with all layers of the epidermis except for the basal layer which was fainter. It stained the outer layers of the hair follicle and the infundibulum. In the eccrine sweat gland it stained the lumen of the straight and coiled intradermal ducts. There was no specific reaction with the apocrine glands or ducts although occasional non-specific light staining was seen.

CAM 5·2 marked the cells of the secretory coil of eccrine glands without any staining of duct cells (Fig. 1). The staining was cytoplasmic with some concentration towards the luminal surfaces. Apocrine glands and ducts were also stained, but rather less strongly (Fig. 2). No other skin structures reacted with this antibody.

HMFG1 marked apocrine glands very faintly but stained the secretions strongly. The sebaceous glands showed very strong membrane labelling with this antibody.

E29.68 marked the intercellular canaliculi of the eccrine secretory coil with great specificity (Fig. 3); no other region of eccrine glands was stained. Apocrine gland secretions and sebaceous glands also stained with this antibody (Fig. 4).

CEA marked the luminal surface of the intraepidermal duct and both the straight and coiled portions of the intradermal duct (Fig. 5). It also demonstrated the intercellular canaliculi of the eccrine secretory coil. There was no staining of the apocrine glands.

Carbonic anhydrase gave no specific staining of any sweat gland structures.

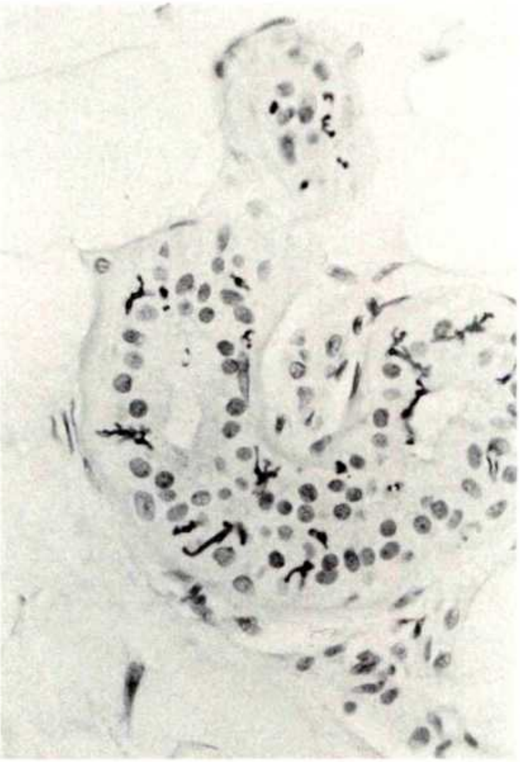

FIGURE 3. E29.68 antibody staining of eccrine sweat gland coil and intercellular canaliculi.

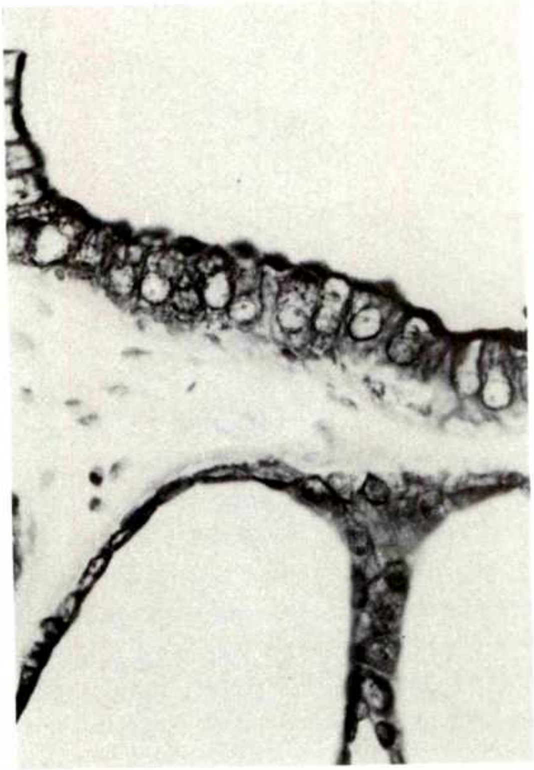

FIGURE 4. E29.68 antibody staining of apocrine glands and secretions.

Brush border antibody gave very weak staining of straight and coiled portions of the intradermal duct and a similar staining of the apocrine glands and ducts.

S-100 marked the cells of the secretory coil faintly. It also stained melanocytes and presumed Langerhans cells but no other skin components. None of the antibodies marked the outer layers of cells of the eccrine duct or coil and we could see no specific staining of myoepithelial cells with these markers or with the actin, myosin and myoglobin antibodies tested.

## DISCUSSION

The eccrine sweat gland develops as a downward growth of the epidermis at 12–16 weeks of intra-uterine life. The development of the intra-epidermal portion, the acrosyringium, occurs by lysosomal lysis of the solid core as distinct from the intra-dermal portion where the cells separate without lysis (Hashimoto, Gross & Lever, 1965, 1966). The secretory coil consists of an outer layer made up of two distinct cell types. The more numerous of these are the serous or 'light' cells which extend through the whole wall of the secretory coil. The 'dark' or mucous cells are fewer in number and are located around the lumen. The terms 'light' and 'dark' refer to light microscopic appearances; on electron microscopy the relative densities of the cells are reversed. Specialized channels exist between the cells, termed intercellular canaliculi. Sweat is produced in the secretory coil as essentially an ultra-filtrate of plasma. It then passes through the coiled

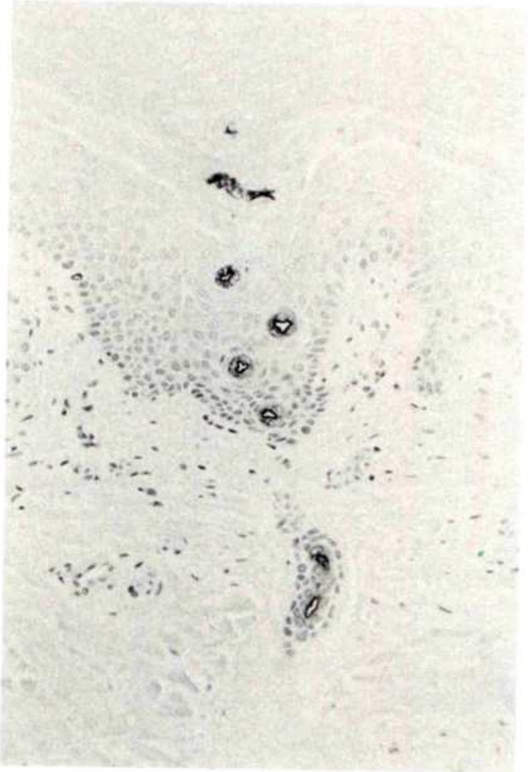

FIGURE 5. CEA antibody staining of intra-epidermal and intra-dermal eccrine sweat gland ducts.

duct, the straight duct and the acrosyringium to the epidermal surface. During passage through the intra-dermal duct the sweat is modified by selective resorption of sodium, mainly within the coiled duct. The coiled duct and the straight duct have similar structures and consist of a single type of luminal cell surrounded by one or two layers of basal cells. The intra-epidermal portion of the duct is lined by dividing and keratinizing cells that can be distinguished from other keratinocytes on electron microscopy. These in turn are surrounded by one or two layers of keratinocytes distinguishable only by their arrangement.

The apocrine gland is simpler and is divided into a secretory portion and a duct. The cells of the secretory region are all of one type and sit on myoepithelial cells or directly on the basement membrane. Secretion is mainly by decapitation and no subsequent modification occurs. The sweat is said to be propelled up the duct by myoepithelial cell contraction and the ducts enter the hair follicle infundibulum above the sebaceous glands. Numerous dense and light granules as well as lipid granules can be seen on electron microscopy and these presumably represent secretory products. The duct consists of three layers of cells whose functions remain obscure.

The pattern of immunoperoxidase staining reflects the morphological and functional subdivisions of the sweat glands. The acrosyringium and the ductal structure in the overlying stratum corneum, which is presumably derived from the acrosyringium, both showed positive staining for CEA (Fig. 5) in marked contrast to the rest of the epidermis. The presence of CEA in human sweat and in eccrine sweat glands has been reported previously (Penneys, Nadji &

McKinney, 1981; Penneys, Nadji & Morales, 1982) and these authors have suggested that CEA may play a role in protection of the sweat gland lumen. In the epidermis the positive staining for CEA seemed to be associated very specifically with the acrosyringium and the overlying keratin structure associated with it and did not appear to be simple contamination from CEA dissolved in sweat. The intradermal duct, both straight and coiled segments, also showed the presence of luminal CEA whilst the secretory coil showed no staining. A similar, highly specific association of an immunological marker with a distinct morphological unit was seen in the staining of the intercellular canaliculi of the secretory coil by E29.68 while the closely related HMFG1, both being milk fat globule antibodies, did not react. A similar pattern of staining of intercellular canaliculi by a milk fat globule antibody has been reported by Graham *et al.* (1985). The epidermal keratin antibody, serum 29, marked the lumen of the intradermal eccrine duct whilst the related hair derived antibody, serum 28, did not. Serum 29 also stained the eccrine intradermal duct lumen and the cells of the intra-epidermal duct, including the specialized area of the stratum corneum demonstrated by CEA staining. In the case of CAM 5·2 which is a cytokeratin monoclonal antibody, the secretory coil was very clearly marked but the adjacent coiled duct showed no reaction (Fig. 1). In the apocrine gland, CAM 5·2 and the two milk fat globule antibodies HMFG1 and E29.68 stained all cells as well as the secretions they contained (Fig. 4), a totally different pattern from that seen in eccrine glands. The presence of active secretion and sodium resorption processes in the eccrine duct suggests the possible involvement of carbonic anhydrase, but the antibody to carbonic anhydrase C did not react. Carbonic anhydrase activity has been demonstrated in human eccrine glands (Briggman, Tashian & Spicer, 1983) but it may be a different iso-enzyme. Comparison is often made between the eccrine gland and the unit of glomerulus and tubule in the kidney but we found only a very weak and rather non-specific reaction with brush border antibody.

If the markers described here are preserved following neoplastic transformation, the panel of antibodies we have used would appear to be sufficiently specific, as a group, to distinguish epidermal tumours arising from any sweat gland source, and also to distinguish these from metastatic tumours even when these arise from tissues as similar as breast (Cotton & Fairris, 1985).

Tumours that lose the markers typical of their normal tissue counterparts and tumours that develop markers characteristic of otherwise unrelated normal tissues, could not be identified by a single antibody. The virtue of using a battery of markers on every tumour rather than using one hopefully 'specific' marker, is that such problems can to some extent be circumvented. It is unlikely that any given tumour will lose all of its markers and even more unlikely that it will develop a new, consistent, pattern of markers entirely characteristic of some other tumour. If we add to this the morphological, cytological and routine staining characteristics then a battery of immunoperoxidase stains should more frequently reduce than increase confusion.

Reproducibility of staining from one laboratory to another is an important technical point. Differing reports are to be found in the literature regarding the staining of sweat gland structures by antibodies to S-100 protein and we have also noticed differences from batch to batch. This may be related to the fact that S-100 protein has recently been shown to consist of two different subunits with different distributions (Takahashi *et al.*, 1984). Antibodies from different sources, apparently generated in an identical fashion, can give totally opposite results. This has been particularly true of polyclonal keratin antibodies, but is also true of monoclonal antibodies as these are derived from clones which need not be identical. This may account for the large differences in specificity found in human milk fat globule (HMFG) antibodies, many of which are generated from identically prepared antigens.

In addition, there is the effect produced by variations in the processing techniques, the most obvious of which are the methods of fixation and paraffin embedding. The nature of the fixative is known to alter the reactivity of various antigens, and prolonged fixation in formalin progressively masks many sites that can be 'reactivated' by partial digestion with proteolytic enzymes; a technique that we have used routinely in the present work. This would seem to argue for the routine use of frozen sections. However, in our experience many antibodies give 'cleaner' results in parafffin, presumably due to the masking of some cross-reacting antibodies, and the better morphology of paraffin sections is so great an advantage as to outweigh the occasional limitations. Similarly, although some monoclonal antibodies, such as those used for T-cell membrane marker identification, do not work well in paraffin sections, many do, or can be persuaded to by adjustment of fixation techniques and proteolytic digestion times. A large amount of pathological research is carried out retrospectively on file material and this is routinely available only as paraffin blocks. Since this is likely to remain the case until we have accumulated large stores of freshly frozen material, the most useful antibodies are likely to be those whose reactions in paraffin sections are well characterized.

Thus, it is necessary at present to test each batch of antibody against a well characterized set of normal tissues and reference tumours, and each laboratory must establish confidence in its individual antibody screen. Universally standardized reference antibodies are not yet available.

## ACKNOWLEDGMENTS

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# Lectin-binding sites in eccrine sweat gland tumours

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## SUMMARY

Lectin-binding sites in sections of formalin-fixed, paraffin-embedded eccrine sweat gland tumours were investigated using fluorescein isothiocyanate (FITC) conjugated peanut agglutinin (PNA), FITC conjugated *Ricinus communis* 1 agglutinin (RCA-1) and FITC conjugated wheat germ agglutinin (WGA). In 22 benign eccrine sweat gland tumours, lectin-binding sites were noted primarily on the cell surface, and in the secretions. In five malignant sweat gland tumours, all showed cytoplasmic lectin-binding sites in variable proportions of malignant cells in addition to cell surface staining. These results indicate that cytoplasmic lectin-binding sites may be a useful marker of neoplastic transformation of eccrine sweat gland tumours.

Lectins are sugar binding proteins or glycoproteins of non-immune origin (Kocourch & Horejsiv, 1981). It has been shown that these lectins recognize complex carbohydrate structures in glycoproteins, glycopeptides and glycolipids of the cell membrane.

Neoplastic transformation has been shown to be accompanied by a variety of changes in the cell membrane (Bramwell & Harris, 1978; Burridge, 1976), many of which are known to involve glycoproteins. A number of tumours have been studied with the view that changes in lectin-binding sites could possibly indicate the invasive potential of tumours (Cummings, 1980) or the risk of recurrence after primary treatment (Furmanski *et al.*, 1981).

Changes in peanut agglutinin (PNA)-binding sites in benign and malignant breast lesions were described by Howard, Ferguson and Batsakis (1981) and Newman, Klein and Rudland (1979). They stated that cytoplasmic PNA-binding sites were present in malignant breast tumours whereas benign breast tumours had PNA-bindings sites localized primarily on the cell surface. This indicates that cytoplasmic PNA-binding sites could be due to neoplastic transformation (Howard *et al.*, 1981; Newman *et al.*, 1979). However, in skin tumours, identical

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TABLE 1. Summary of lectin-binding in eccrine sweat gland tumours[a]

| | | Tumours (No.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Benign eccrine sweat gland tumours (22) | | | | | Malignant eccrine sweat gland tumours (5) | | |
| Lectins | Structure stained | Syringoma (5) | Clear cell hidradenoma (5) | Eccrine poroma (6) | Eccrine spiradenoma (2) | Mixed tumour of the skin (4) | Malignant eccrine poroma (1) | Eccrine duct carcinoma (3) | Eccrine gland carcinoma (1) |
| PNA | Cytoplasm | 0 | 0 | 0 | 0 | 0–2+[b] | 4+ | 0–4+[c] | 3+ |
| | Plasma membrane | 0–1+ | 4+ | 2+ | 3+ | 3+ | 4+ | 3–4+ | 3+ |
| RCA-1 | Cytoplasm | 0 | 0 | 0 | 0 | 0–1+[b] | 2+ | 0–2+[c] | 2+ |
| | Plasma membrane | 0–1+ | 1+ | 2+ | 3+ | 1+ | 2+ | 1–2+ | 2+ |
| WGA | Cytoplasm | 0 | 0 | 0 | 0 | 0–2+[b] | 4+ | 4+[c] | 4+ |
| | Plasma membrane | 3+ | 4+ | 2+ | 3+ | 4+ | 4+ | 4+ | 4+ |

[a] Results are expressed as intensity of staining as evaluated by immunofluorescent microscopy. Grading performed on the following scale: 1+, faint; 2+, moderate; 3+, intense; 4+, very intense.

[b] One case showed focal cytoplasmic staining with FITC-PNA, FITC-RCA-1 and FITC-WGA.

[c] One case showed very intense cytoplasmic staining in a very small number of tumour cells with FITC-WGA but not with other lectins.

surface lectin-binding sites have been reported in squamous cell carcinoma, on keratinocytes in basal cell epithelioma (Uno *et al.*, 1983) and melanocytes in malignant melanoma (Romani, Schuler & Fritsch, 1983). It therefore remains to be shown whether this will have practical value in diagnosis or prognosis (Ponder, 1983).

In a previous paper, we demonstrated the presence of lectin-binding sites on the cell surface and in the secretions of normal eccrine glands using fluorescein isothiocyanate (FITC), conjugated peanut agglutinin (PNA) and FITC conjugated wheat germ agglutinin (WGA) (Tamaki *et al.*, 1985). These results prompted us to investigate the presence and distribution of lectin-binding sites in eccrine sweat gland tumours.

In this paper, we demonstrate that cell surface lectin-binding sites are present in benign eccrine sweat gland tumours. In contrast, both cell surface and cytoplasmic lectin-binding sites are present in malignant eccrine sweat gland tumours using FITC-PNA, FITC conjugated *Ricinus communis* 1 agglutinin (RCA-1) and FITC-WGA. The cytoplasmic lectin-binding sites may be the result of malignancy-associated cytostructural glycoconjugate alterations in eccrine sweat gland tumours.

## METHODS

Twenty-seven cases of benign and malignant eccrine sweat gland tumours were selected from the files of our department. All tissues had been fixed routinely in buffered formalin and embedded in paraffin. The histological findings in these tumours are presented in Table 1. The diagnosis was based on the results of haematoxylin and eosin (H&E) staining, enzyme histochemistry and electron microscopy. H&E sections were reviewed to confirm the diagnosis.

Conjugates of fluorescein isothiocyanate (FITC) with PNA, RCA-1 and WGA were obtained from E-Y Laboratories (San Mateo, CA, U.S.A.). *Vibrio cholerae* neuraminidase, 1 IU/ml, was obtained from Behringwerke AG (Marburg, F.R.G.).

Paraffin sections were deparaffinized and rehydrated and washed three times for 15 min in phosphate buffered saline (PBS), pH 7·4. FITC-PNA and FITC-WGA were used at a dilution of 1:40 (25 μg/ml) and FITC-RCA-1 was used at a dilution of 1:10 (50 μg/ml).

Tissue sections to be pre-treated with neuraminidase were overlaid with neuraminidase (1 IU/ml) at room temperature for 30 min. Tissue sections, both treated with neuraminidase and untreated, were then exposed to FITC-lectin conjugates for 30 min at room temperature in a

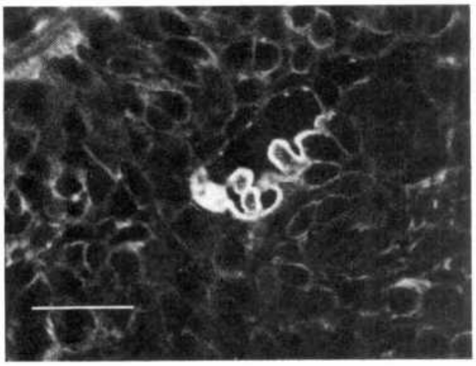

FIGURE 1. Clear cell hidradenoma: PNA-binding sites are seen on the cell surface. They are more intense along the luminal portion of the cells. (Original × 1320.) Bar = 10 μm.

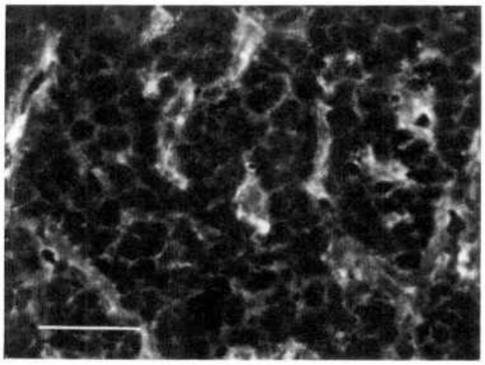

FIGURE 2. Eccrine spiradenoma: WGA-binding sites are seen on the cell surface and along the luminal portion of the cells. (Original × 1320.) Bar = 10 μm.

moist chamber. In the control sections the following FITC-lectin conjugates were used: FITC-PNA in 0·1 M galactose; FITC-WGA in 0·2 M N-acetylglucosamine and FITC-RCA-1 in 0·2 M lactose.

## RESULTS

The benign eccrine sweat gland tumour group included five syringomas, five clear cell hidradenomas, six eccrine poromas, two eccrine spiradenomas, and four mixed tumours of the skin. The results are summarized in Table 1. Lectin-binding sites were present primarily on the cell surface and in the secretions. More intense cell surface staining occurred on the luminal side of the cells. The intensity of fluorescence was different for each tumour. WGA-binding sites had the strongest staining in most of the tumours, and RCA-1-binding sites tended to have the weakest staining.

In syringoma, WGA-binding sites were clearly observed on the luminal side of the cells and in the secretions, whereas RCA-1 and PNA binding sites were hardly visible. In clear cell hidradenoma, PNA-binding sites were noted on the cell surface with more intense surface staining on the luminal side (Fig. 1). The secretions contained bright WGA-binding sites. In

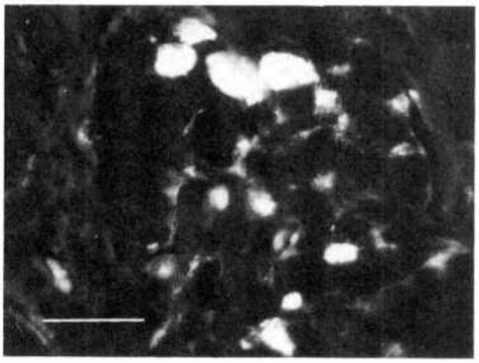

FIGURE 3. Eccrine duct carcinoma: PNA-binding sites are seen in the cytoplasm of malignant cells as well as on the cell surface. (Original × 1320.) Bar = 10 μm.

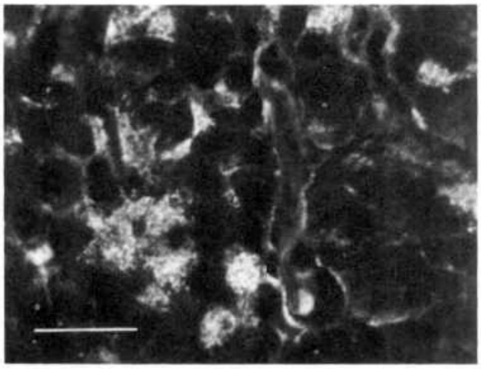

FIGURE 4. Eccrine duct carcinoma: WGA-binding sites are noted in the cytoplasm as well as on the cell surface. (Original × 1320.) Bar = 10 $\mu$m.

eccrine poroma and in eccrine spiradenoma the lectin-binding sites had a similar fluorescent intensity and cell surface staining pattern (Fig. 2). However, the secretions were hardly visible in eccrine poroma. In mixed tumours of the skin, most cells had lectin-binding sites on the cell surface. However, in one of the four cases of mixed tumour of the skin, additional focal cytoplasmic staining was noted. This appeared to be at the part of the tumour showing tubular and cystic partially branching lumina. There is still some debate as to whether these cells are apocrine or eccrine in type (Lever & Schaumburg-Lever, 1983). Our histochemical findings show that some of these cells have lectin-binding sites similar to apocrine glands rather than eccrine glands. Pre-treatment with neuraminidase increased the intensity of fluorescence of PNA-binding sites, but did not change RCA-1 or WGA binding sites.

In the controls, the binding specificity for a distinct sugar was tested as previously described (Tamaki *et al.*, 1985). FITC-PNA was inhibited by 0·1 M galactose, FITC-RCA-1 was inhibited by 0·2 M lactose, and FITC-WGA was inhibited by 0·2 M N-acetylglucosamine.

The malignant eccrine sweat gland tumour group comprised one malignant eccrine poroma, three eccrine duct carcinomas and one eccrine gland carcinoma. The results are summarized in Table 1. In all malignant tumours, cell surface staining was observed, and was particularly intense along the luminal aspect in a pattern similar to the benign tumours. In addition,

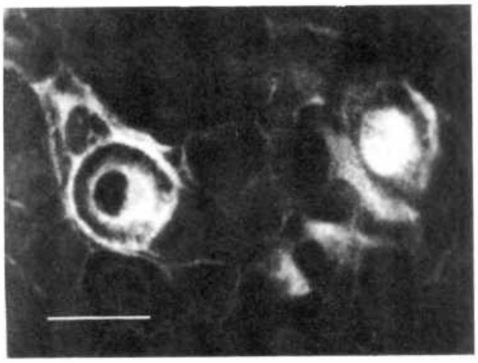

FIGURE 5. Malignant eccrine poroma: WGA-binding sites are seen in the cytoplasm. (Original × 1320.) Bar = 10 $\mu$m.

however, all the cases had cytoplasmic lectin-binding sites in a variable proportion of the malignant cells. The brightness of staining and localization of the cytoplasmic lectin-binding sites differed in each case. Cytoplasmic staining was mainly noted in morphologically malignant cells (Figs 3–5). In one case of eccrine duct carcinoma, cytoplasmic staining occurred only with WGA in a very small number of cells.

In control sections, the binding specificity for a distinct sugar was proved; FITC-PNA was inhibited by 0·1 M galactose, FITC-RCA-1 was inhibited by 0·2 M lactose, and FITC-WGA was inhibited by 0·2 M N-acetylglucosamine.

Pre-treatment with neuraminidase increased the intensity of fluorescence and the number of positive cells with PNA-binding sites, but had no effect on the RCA-1 or WGA binding sites.

## DISCUSSION

Although we could not investigate the lectin-binding sites of intermediate tumour types and on frozen sections, the cytoplasmic lectin-binding sites seen in malignant eccrine sweat gland tumours were in sharp contrast to the exclusively cell surface staining seen in benign eccrine sweat gland tumours.

Howard *et al.* (1981) showed that 17 out of 22 cases of breast carcinoma contained cytoplasmic PNA-binding sites, whereas benign breast lesions had PNA-binding sites on the luminal portion of the membrane and/or in the intraluminal secretions. The five cases of breast carcinoma which had no PNA-binding sites appeared the most poorly differentiated when evaluated by histological criteria. Newman *et al.* (1979) have also noted cytoplasmic PNA-binding sites in differentiated rather than in undifferentiated breast carcinoma. Recently, Franklin (1983) has shown the presence of WGA, RCA-1 and PNA-binding sites in the cytoplasm of breast carcinoma. They also found that WGA-binding sites were the brightest and most consistent in breast carcinoma.

Our histochemical findings in eccrine sweat gland tumours are very similar to the findings of Franklin in breast lesions (Franklin, 1983), although they found RCA-1 to be the second most strongly bound lectin in contrast to our finding of PNA.

Penneys *et al.* have found carcinoembryonic antigen (CEA) in both benign (Penneys, Nadji & Morales, 1982a) and malignant eccrine sweat gland tumours (Penneys *et al.*, 1982b). CEA has been shown to be rich in WGA-binding sites (Chism *et al.*, 1977). Therefore, it is not surprising that WGA-binding sites were found in these tumours. Our study has extended the previously reported CEA reactivity in these tumours to WGA-binding sites and demonstrated that different patterns of WGA-binding sites are present in benign and malignant eccrine sweat gland tumours.

WGA binds preferentially to $\beta$-N-acetyl-D-glucosamine and/or sialic acid, independent of their positions within an oligosaccharide chain (Bhavanandan & Katlic, 1979). Pre-treatment of the eccrine sweat gland tumours with neuraminidase did not influence their WGA-binding sites, indicating that the main receptor of WGA in eccrine sweat gland tumours is not sialic acid.

RCA-1-binding sites showed a weaker but similar staining pattern to WGA-binding sites in both benign and malignant eccrine sweat gland tumours.

The distribution of PNA-binding sites was also similar to the WGA and RCA-1 binding sites. PNA was as strongly bound as WGA in most of the malignant tumours. PNA has been shown to bind $\beta$-D-galactosyl (1–3)-N-acetyl-D-galactosamine which is purported to be the antigenic structure of Thomsen-Friedenreich antigen (T antigen) (Lotan *et al.*, 1975). Pre-treatment with neuraminidase increased the intensity of fluorescence of the PNA-binding sites as well as the number of positive cells. This may be because the PNA-binding sites were masked, either directly by sialic acid, or indirectly by sialic acid residues on neighbouring oligosaccharide chains, which modify the steric and electrostatic conditions of the cell membrane (Maylie-Pfenninger & Jamieson, 1980).

Very recently, Ookusa reported that in eccrine sweat glands RCA-1 and WGA-binding sites were found on the cell surface and in the cytoplasm but no PNA-binding sites were detected (Ookusa, 1984). The difference between their results and ours may be due to technical differences. We used neuraminidase but they did not, and we used a direct fluorescence method using FITC conjugated lectins whereas they used an indirect method using biotin-conjugated lectins and avidin-conjugated horseradish peroxidase.

It has been shown that purified T antigen elicits delayed type hypersensitivity reactions in patients with breast carcinoma, but not in healthy controls or patients with benign breast disease (Springer, Desai & Scanlon, 1976). It has also recently been shown that WGA enhances macrophage mediated *in vitro* lysis of syngeneic mammary tumour cells (Kurisu, Yamazaki & Mizuno, 1980).

These results suggest that cytoplasmic lectin-binding sites in eccrine sweat gland tumours may be a useful marker for neoplastic transformation. The presence of lectin-binding sites in malignant eccrine sweat gland tumours may facilitate the diagnosis and immunotherapy of these tumours.

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# Expression of gamma-glutamyl transpeptidase in normal and neoplastic epithelial cells of human skin: a marker for distinguishing malignant epithelial tumours

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## SUMMARY

The distribution of gamma-glutamyl transpeptidase (GGT) activity was studied histochemically in benign and malignant epithelial tumours of human skin. We found that GGT activity in normal skin was confined to the secretory portion of the eccrine and apocrine glands and to the inner root sheath of the hair follicles. In Bowen's disease and actinic keratosis, GGT activity was noted focally in areas where atypical cells were observed. In extramammary Paget's disease, GGT activity was found only in large round cells scattered among GGT negative epidermal cells. No GGT activity was observed in basal cell epitheliomas or benign epithelial tumours, while squamous cell carcinoma and eccrine porocarcinoma exhibited intense GGT activity. Our study suggests that GGT may be useful as a histochemical marker for distinguishing malignant tumours from benign epithelial tumours in human skin.

Gamma-glutamyl transpeptidase (GGT) is a membrane-bound enzyme which catalyses the transfer of the gamma-glutamyl moiety of glutathione and other gamma-glutamyl compounds to amino acids (Szewczuk & Baranowski, 1963).

Histochemical studies of rodent skin have shown that the epidermis of adult rodents does not exhibit GGT activity, but activity was detected in foetal epidermis and in foetal and adult hair follicles (Buxman, Kocarnik & Holbrook, 1979). De Young *et al.* (1978) observed that chemically-induced papillomas in animals were GGT negative, while most chemically-induced squamous cell carcinomas exhibited GGT activity. Klein-Szanto *et al.* (1983) demonstrated that not only were chemically-induced squamous cell carcinomas GGT positive, but also some histologically benign papillomas contained several GGT positive areas. On the basis of these observations, the authors suggested that GGT might prove to be a good indicator of conversion

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from benign to malignant conditions in experimental skin carcinogenesis. We have investigated GGT activity in benign and malignant epithelial tumours in human skin.

## METHODS

### *Skin tumours*

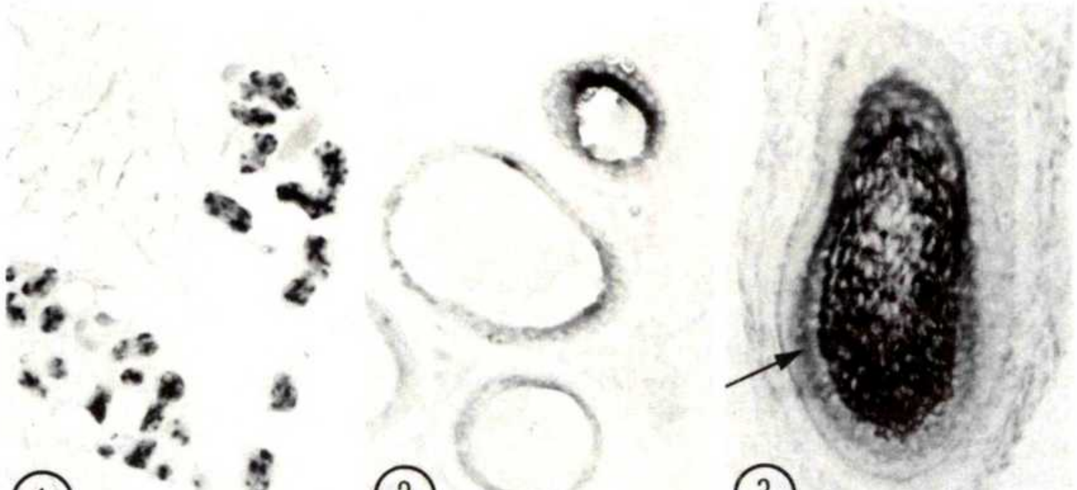
Skin tumours were excised from 46 patients: squamous cell carcinoma (SCC), 4; basal cell epithelioma (BCE), 7; Bowen's disease, 12; extramammary Paget's disease, 2; actinic keratoses, 2; keratoacanthoma, 2; seborrhoeic keratoses, 10; epidermal cyst, 5; epidermal naevi, 2; eccrine porocarcinoma, 1; eccrine poroma, 3; eccrine duct epithelioma, 1; sebaceous epithelioma, 1; and trichoepithelioma, 3.

A portion of each tumour was processed for routine histological examination and the remainder was used to prepare frozen sections for GGT histochemistry.

### *GGT histochemistry*

Frozen sections (6 μm) were cut using a cryostat. Sections were fixed in cold acetone for 1 h and incubated in a freshly prepared solution containing γ-glutamyl-4-methoxy-β-naphtlylamide as substrate and glycylglycine as acceptor for 30 min according to the method of Rutenburg *et al.* (1969). After rinsing with solutions of 0·85% NaCl, 0·1 M $CuSO_4$ and 0·85% NaCl, the sections were counterstained with haematoxylin and then mounted in glycerol.

Sections incubated in substrate-free solution and sections from heat-inactivated tissue were used as controls.



Histochemical staining for GGT.

FIGURE 1. Eccrine glands. Intense GGT activity in the secretory portion. (Original × 200).

FIGURE 2. Apocrine glands. GGT activity in the secretory portion. (Original × 320).

FIGURE 3. Hair follicle. GGT activity in the inner root sheath of anagen hair follicle (arrow). (Original × 320).

## RESULTS

### *GGT histochemistry of normal skin*

GGT activity was examined in the normal skin adjacent to tumour lesions. No GGT activity was detected in normal epidermis. Intense GGT activity was present in the secretory portion of eccrine and apocrine glands, but not in the ductal portion. (Figs 1 and 2). The inner root sheath of anagen hair follicles also exhibited GGT activity (Fig. 3).

### *GGT histochemistry of benign epithelial tumours*

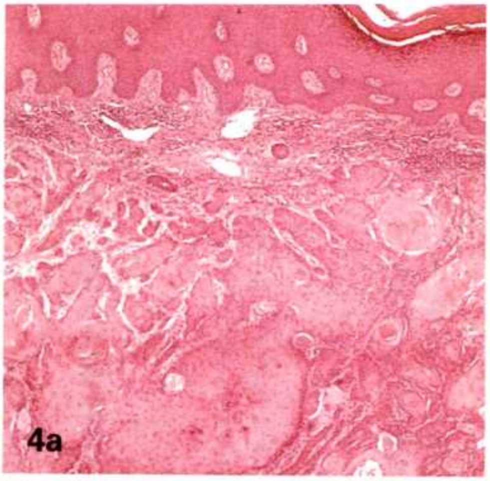
No GGT positive areas were detected in any of the benign tumours of keratoacanthoma, seborrhoeic keratosis, epidermal cyst and epidermal naevus, or in those of eccrine poroma, eccrine duct epithelioma and sebaceous epithelioma. Trichoepithelioma exhibited weak GGT activity around the horn cyst.

### *GGT histochemistry of pre-malignant and malignant epithelial tumours*

The histochemical findings are summarized in Table I. All four cases of SCC showed intense GGT activity. There was a direct relationship between the grade of tumour differentiation and the intensity of GGT activity. GGT activity was observed in the tumour cells of SCC, but not in the normal epidermis overlying the tumours (Fig. 4a, b). In Bowen's disease, 10 of 12 lesions contained focal GGT positive areas in which the presence of cell atypia was confirmed by serial haematoxylin and eosin stained sections (Fig. 5a, b). All cases of actinic keratosis showed focal GGT positive areas. In extramammary Paget's disease, GGT activity was expressed only in large, round cells scattered among GGT negative epidermal cells (Fig. 6a, b). Eccrine porocarcinoma exhibited regional differences in the intensity of GGT activity. No positive GGT activity was noted in any of the basal cell epitheliomas.

TABLE I. GGT activity in human epithelial tumours

| Histological diagnosis | Total | GGT positive |
|---|---|---|
| Benign | | |
| Keratoacanthoma | 2 | 0 |
| Seborrhoeic keratosis | 10 | 0 |
| Epidermal cyst | 5 | 0 |
| Epidermal naevus | 2 | 0 |
| Eccrine poroma | 3 | 0 |
| Eccrine duct epithelioma | 1 | 0 |
| Sebaceous epithelioma | 1 | 0 |
| Trichoepithelioma | 3 | 1 |
| Pre-malignant | | |
| Actinic keratosis | 2 | 2 |
| Malignant | | |
| Squamous cell carcinoma | 4 | 4 |
| Bowen's disease | 12 | 10 |
| Extramammary Paget's disease | 2 | 2 |
| Basal cell epithelioma | 7 | 0 |
| Eccrine porocarcinoma | 1 | 1 |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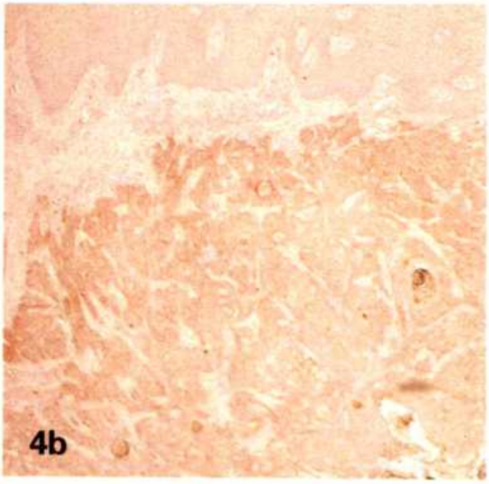
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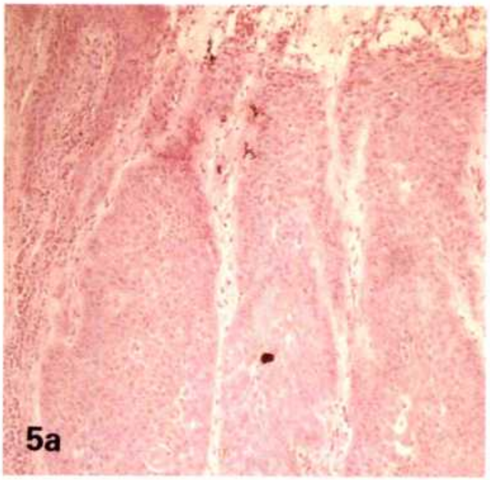
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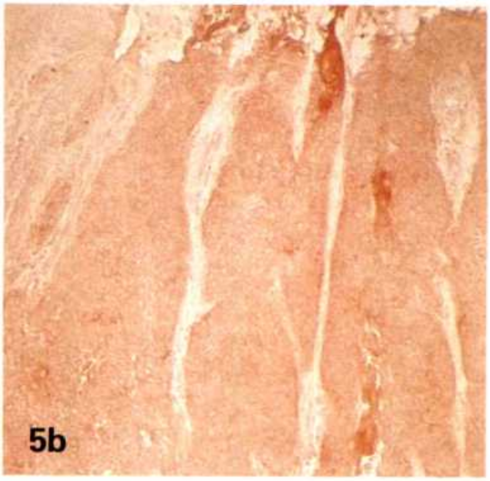
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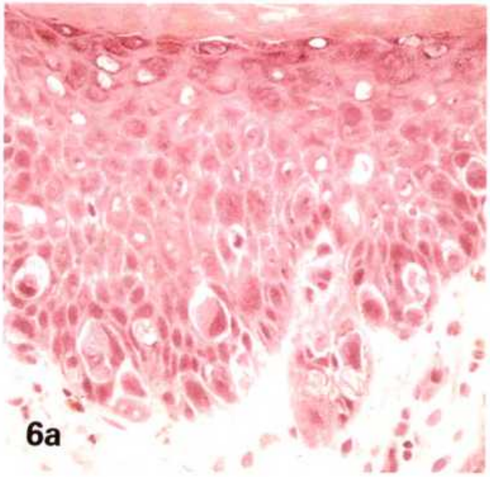
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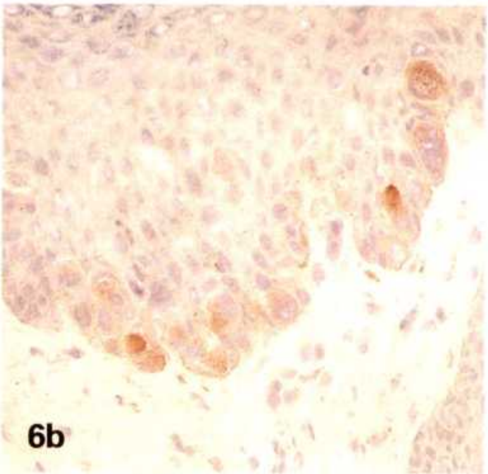
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## DISCUSSION

This study demonstrated clearly that GGT activity is expressed intensely in pre-malignant and malignant epithelial tumours of human skin, irrespective of their origin.

In biochemical studies of human tissue, prominent GGT activity has been detected in kidney, pancreas and liver (Goldbarg *et al.*, 1960) and in prostate tissues (Rosalki & Rowe, 1973), the highest activity being seen in tissues from the kidney. GGT activity has also been observed in spleen, lung, bowel, placenta and thyroid homogenates (Goldbarg *et al.*, 1960). Cell fractionation of kidney and liver tissues has indicated that GGT exists in two forms, a minor soluble form of low molecular weight in the supernatant and a major membrane-bound form of high molecular weight in the microsomal fraction (Szewczuk, 1966). Histochemically, prominent GGT activity has also been noted in the brush border of the epithelial cells lining the tubules of the kidney and in the luminal border of epithelial cells lining the bilary ducts of the liver (Albert, Orlowski & Szewczuk, 1961; Albert, Orlowska & Orlowski, 1964). It has also been proposed that the enzyme is involved in the transport of some amino acids across the epithelial membrane through the $\gamma$-glutamyl cycle (Orlowski & Meister, 1970; Meister, 1973).

In human malignant tumours, high GGT activity has been observed histochemically in hepatoma (Gerber & Thung, 1980), colon cancer (Fiala *et al.*, 1979), lung cancer (Groscurth, Fleming & Kistler, 1977) and pancreatic cancer (Yamaguchi, Sugimoto & Kawai, 1984). Increased GGT activity has also been reported in experimental carcinogenesis of these organs in animals (Moriyama, Kawaoi & Hirota, 1983; Kalengayi & Desmet, 1978; Solt, 1981; Fiala, Fiala & Dixon, 1972). In animal skin, Buxman *et al.* (1979) demonstrated that GGT activity is present in foetal rat epidermis but not in adult rat epidermis. De Young *et al.* (1978) and Klein-Szanto *et al.* (1983) showed that GGT is expressed in chemically-induced mouse SCC. These findings led us to an investigation of human skin tumours and from this study it has become evident that GGT is also expressed in malignant epithelial tumours of human skin. GGT activity, however, was also observed in the secretory portion of eccrine and apocrine glands and in the inner root sheath of anagen hair. This expression of GGT activity in the secretory portion of sweat glands and hair follicles suggests that the enzyme may be involved in some aspect of the differentiation of the epithelial cells of the skin appendages. The finding that GGT activity was not expressed in BCE may support the suggestion by Lever & Shaumburg-Lever (1983) that BCE is not a carcinoma but a naevoid tumour or a hamartoma derived from primary epithelial germ cells. However, we were not able to determine whether the re-induction of GGT activity in malignant epithelial cells reflects a tendency towards reversion to a foetal phenotype with increasing malignancy, whether active GGT is required for the metabolic changes related to malignant transformation, and whether high GGT activity is caused by factors modulating enzyme expression during malignant transformation.

Recently, Turbitt & MacKie (1981) described a significant loss of HLA antigen related $\beta_2$-microglobulin from the cell surface of cutaneous pre-malignant and malignant lesions. An

FIGURE 4. Squamous cell carcinoma. (a) Haematoxylin and eosin ( × 80), (b) GGT activity in tumour cells, but not in the normal epidermis overlying the tumour ( × 80).

FIGURE 5. Bowen's disease (a) Haematoxylin and eosin, ( × 200), (b) focal GGT positive area in which the presence of cell atypia was confirmed by serial H & E-stained sections ( × 200).

FIGURE 6. Extramammary Paget's disease. (a) Haematoxylin and eosin ( × 200), (b) GGT activity in large round cells scattered among GGT negative epidermal cells ( × 200).

alteration in lectin binding capacity during malignant conversion of epithelial tumours of skin has also been reported (Louis *et al.*, 1981; Græm, 1982). GGT may also be useful as a marker for diagnosing malignant skin tumours. Further studies of GGT may also provide information concerning metabolic changes in the epithelial cells during carcinogenesis.

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# HLA-DR expression on keratinocytes is a common feature of diseased skin

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## SUMMARY

Biopsy specimens from 185 patients with 52 different skin disorders were investigated by indirect immunofluorescence staining for the presence of HLA-DR bearing keratinocytes and their association with an underlying inflammatory infiltrate and in particular with activated (HLA-DR-positive, Leu-4-positive) T lymphocytes. HLA-DR expression on keratinocytes was demonstrated in 38 dermatoses, including lymphocytic vasculitis, lupus erythematosus, morphea, vitiligo, lichen planus, cutaneous T-cell lymphoma, various infectious dermatoses, allergic contact dermatitis, granulomatous dermatoses, Sweet's syndrome, lichen sclerosus and erythema nodosum. In 27 of these this had not previously been reported. Occurrence of HLA-DR on keratinocytes was invariably linked to the presence of a lymphocytic infiltrate containing numerous activated T-cells (Leu-4 +, HLA-DR +) whereas such infiltrates were not accompanied by HLA-DR expression on keratinocytes in all the dermatoses investigated, as in pseudolymphoma and erythema anulare centrifugum. However, HLA-DR positive keratinocytes were consistently absent in skin disorders lacking any significant lymphocytic infiltration (e.g. leukocytoclastic vasculitis, bullous autoimmune dermatoses, genodermatoses and mastocytosis). Although it has been suggested that HLA-DR-positive keratinocytes are involved in various immune responses of the skin, their exact functional significance is, as yet, unknown.

Although HLA-DR or class II major histocompatibility (MHC) antigens have generally been related to cells of the immune system, recent studies have revealed the presence of HLA-DR antigens on cells not definitely known to serve immunological functions; one of these cell types is the keratinocyte. While in normal skin HLA-DR antigen is confined to Langerhans cells (Stingl, Tamaki & Katz, 1980) and acrosyringial epithelial cells (Murphy *et al.*, 1983), in a number of skin disorders (Aiba & Tagami, 1984; Lampert, 1984; Smolle, 1985) including graft-vs-host disease (Lampert *et al.*, 1982), lichen planus (Tjernlund, 1980), mycosis fungoides (Tjernlund, 1978), allergic contact dematitis (MacKie & Turbitt, 1983) and alopecia areata (Messenger *et al.*, 1984) keratinocytes synthesize (Volc-Platzer *et al.*, 1984) and express HLA-

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TABLE 1. HLA-DR on keratinocytes in various dermatoses

| | No. of patients | |
|---|---|---|
| Dermatosis | Total | HLA-DR-positive keratinocytes |
| Lymphocytic vasculitis | 12 | 12 |
| Lymphomatoid papulosis | 1 | 1 |
| Pityriasis lichenoides | 5 | 5 |
| Purpura pigmentosa | 6 | 6 |
| Lupus erythematosus | 16 | 12 |
| Morphea | 4 | 4 |
| Vitiligo | 3 | 3 |
| Lichen planus | 6 | 5 |
| Cutaneous T-cell lymphoma | 7 | 6 |
| Mycosis fungoides | 4 | 3 |
| Sézary's syndrome | 3 | 3 |
| Infectious dermatoses | 14 | 14 |
| Acrodermatitis chronica atrophicans | 1 | 1 |
| Erythema chronicum migrans | 1 | 1 |
| Epidermomycosis | 1 | 1 |
| Herpes simplex | 2 | 2 |
| Herpes zoster | 3 | 3 |
| Molluscum contagiosum | 1 | 1 |
| Pityriasis rosea | 1 | 1 |
| Tertiary nodular syphilis | 1 | 1 |
| Viral warts | 2 | 2 |
| Scabies norvegica | 1 | 1 |
| Allergic contact dermatitis | 8 | 5 |
| Granulomatous dermatoses | 10 | 9 |
| Granuloma annulare | 5 | 5 |
| Necrobiosis lipoidica | 2 | 1 |
| Sarcoidosis | 3 | 3 |
| Sweet's syndrome | 5 | 4 |
| Lichen sclerosus | 5 | 5 |
| Erythema nodosum | 9 | 5 |
| Leukocytoclastic vasculitis | 15 | 0 |
| Bullous autoimmune dermatoses | 19 | 1 |
| Dermatitis herpetiformis Duhring | 2 | 0 |
| Herpes gestationis | 2 | 0 |
| Pemphigus vulgaris | 5 | 0 |
| Bullous pemphigoid | 10 | 1 |
| Pseudolymphoma | 8 | 1 |
| Erythema annulare centrifugum | 6 | 0 |
| Genodermatoses | 10 | 0 |
| Darier's disease | 1 | 0 |
| Lamellar ichthyosis | 1 | 0 |
| Familial benign pemphigus | 4 | 0 |
| Actinic porokeratosis | 1 | 0 |
| Erythropoietic protoporphyria | 2 | 0 |
| Pseudoxanthoma elasticum | 1 | 0 |
| Psoriasis | 5 | 2 |
| Phototoxic dermatitis | 1 | 1 |
| Solar dematitis | 1 | 1 |
| Fixed drug eruption | 1 | 1 |
| Erythema multiforme | 2 | 1 |
| Graft-vs-host disease | 2 | 2 |
| Mastocytosis | 2 | 0 |
| Melanoma | 3 | 1 |
| Verrucous naevus | 1 | 0 |
| Polymorphous light eruption | 3 | 2 |
| Urticaria | 2 | 1 |
| Nodular vasculitis | 1 | 1 |
| Melanocytic naevi | 4 | 0 |

DR molecules. Thyroid follicular epithelial cells have been reported to bear HLA-DR antigen in Graves' disease, whereas they are HLA-DR-negative in the normal state (Hanafusa *et al.*, 1983). It has been suggested that 'aberrant' expression of MHC class II antigens on these cells might play an important role in the initiation and perpetuation of immune reactions (Bottazzo, Pujol-Borrell & Hanafusa, 1983). Similar views have been voiced regarding the skin (Nickoloff *et al.*, 1985). To determine the frequency and significance of HLA-DR-bearing keratinocytes, we investigated a broad spectrum of skin disorders.

## METHODS

Cryostat biopsy specimens from 185 patients with 52 different skin disorders (Table 1), both inflammatory and non-inflammatory, were subjected to indirect immunofluorescence staining using a monoclonal antibody against human non-polymorphic HLA-DR (Becton-Dickinson).

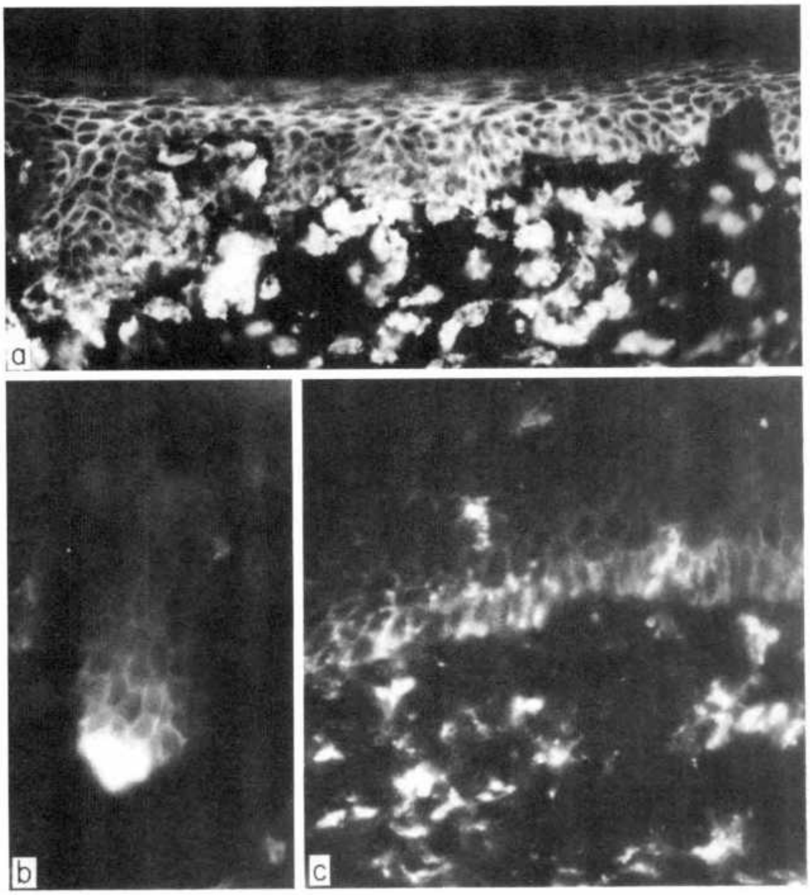

FIGURE 1. Distribution of HLA-DR-positive keratinocytes. Immunofluorescence staining of (a) whole epidermis (lichen sclerosus) (original × 90), (b) focal keratinocyte cluster (vitiligo) (original × 175) and (c) basal epidermis (morphea) (original × 250). Staining patterns varied considerably in serial sections and were not specific for the underlying disease.

In order to identify activated T-lymphocytes within the inflammatory dermal infiltrate by their phenotype, selected specimens were double-stained either with anti-Leu 4 (Becton-Dickinson), which recognizes peripheral T-lymphocytes, and anti HLA-DR, or anti-Leu 4 and TEC-MLR-3 (Bio-Yeda) antibody which recognizes activated T-lymphocytes (Corte *et al.*, 1981). Normal skin obtained at the time of plastic surgery served as a negative control.

*Immunofluorescence*

Unfixed sections were thoroughly washed in PBS containing 1% bovine serum albumin and

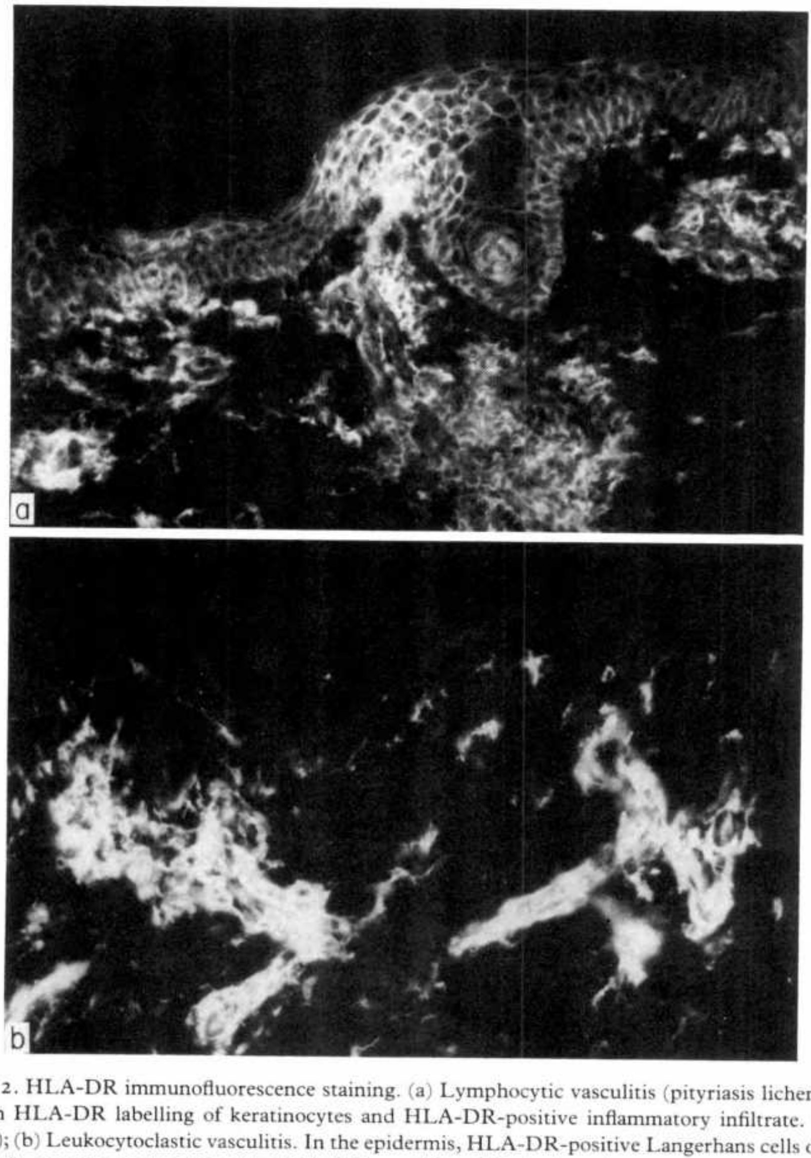

FIGURE 2. HLA-DR immunofluorescence staining. (a) Lymphocytic vasculitis (pityriasis lichenoides). Uniform HLA-DR labelling of keratinocytes and HLA-DR-positive inflammatory infiltrate. (Original × 90); (b) Leukocytoclastic vasculitis. In the epidermis, HLA-DR-positive Langerhans cells only. In the dermis, vascular and perivascular HLA-DR reactivity. (Original × 90).

0·02% sodium-azide and incubated with the 1st antibody (anti HLA-DR or TEC-MLR-3) at a dilution of 1:30 for 30 min at room temperature. After rinsing, the slides were exposed to affinity purified $F(ab')_2$ goat-anti-mouse IgG + IgM rhodamine-conjugate (Grub antibodies, Scandic, Vienna, Austria) at a dilution of 1:40 for 30 min at room temperature. For double labelling studies a blocking solution (mouse Ig, 250 μg/ml) was added for 10 min at room temperature, after the first set of reagents. The 2nd antigen was visualized using anti-Leu 4 fluorescein (FITC) conjugate.

## RESULTS

In 38 of the 52 skin disorders investigated (Table 1), HLA-DR was expressed on the surface of keratinocytes. In 27 disorders, this phenomenon had not previously been described. Several morphological patterns of HLA-DR expression were found; either uniform labelling through-

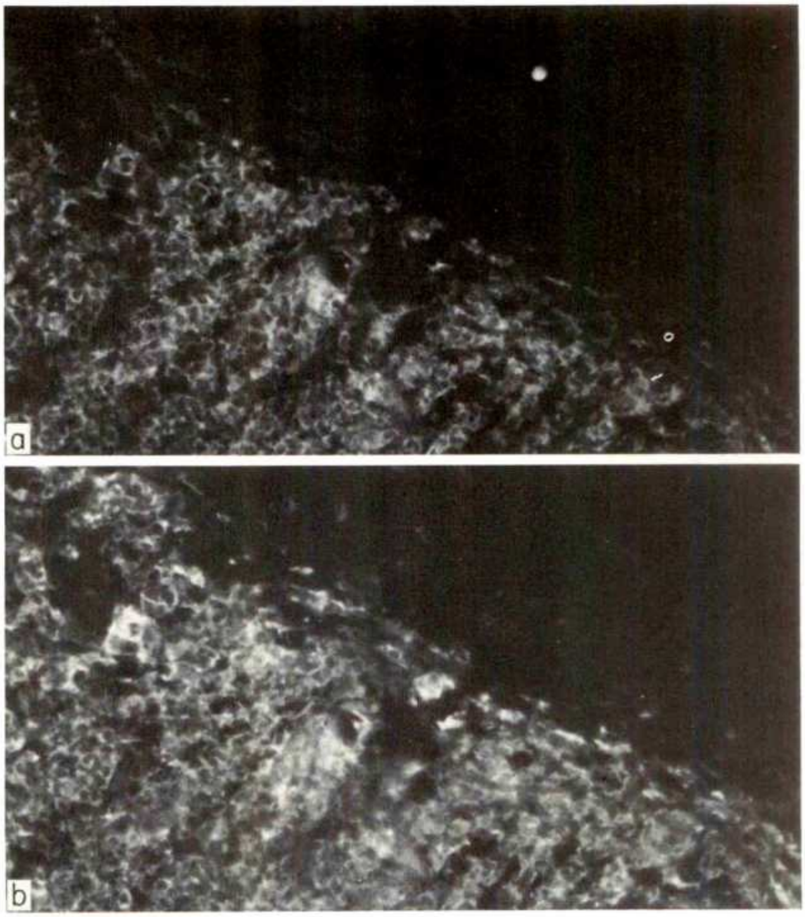

FIGURE 3. Pseudolymphoma. Immunofluorescence double staining with (a) anti Leu-4 and (b) anti HLA-DR antibody. The dermal infiltrate contains numerous activated (Leu-4 +, HLA-DR +) T lymphocytes. However, keratinocytes are clearly HLA-DR negative. (Original × 90).

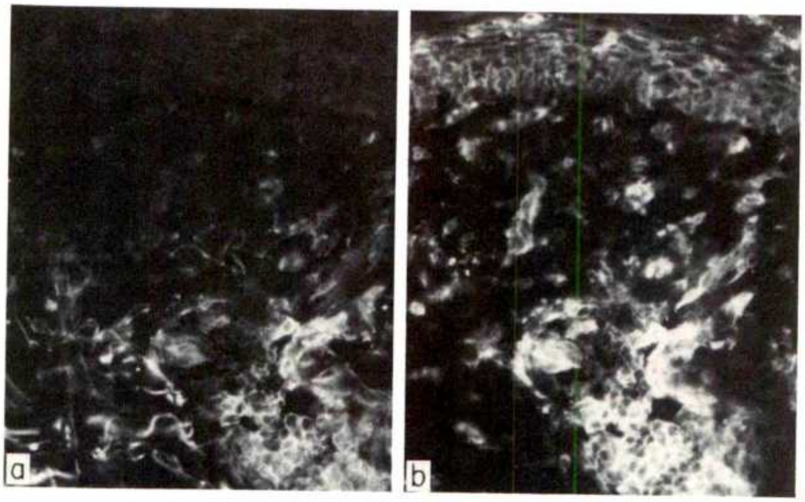

FIGURE 4. Pityriasis lichenoides. Immunofluorescence double staining with (a) anti Leu-4 and (b) anti HLA-DR antibody. The dermal infiltrate contains numerous activated (Leu-4+, HLA-DR+) T lymphocytes. Keratinocytes bear HLA-DR antigen. (Original × 90).

out the epidemis or labelling of clusters or stretches of keratinocytes in the mid-epidermis or in the basal layer (Fig. 1). Staining patterns varied considerably in serial sections and were not specific for the underlying disease. In perilesional and normal control skin keratinocytes did not express HLA-DR.

There appeared to be no simple association between HLA-DR expression and the nature of the dermatosis. Some of the 'autoimmune' disorders were positive (e.g. lupus erythematosus and vitiligo) whereas others were negative (e.g. pemphigus vulgaris and bullous pemphigoid). Most skin disorders linked to immune reactions were positive (e.g. all infectious diseases), a few of them were negative (e.g. leukocytoclastic vasculitis). Some inflammatory diseases of unknown cause were positive (e.g. pigmented purpura), others negative (e.g. erythema anulare centrifugum, pseudolymphoma). HLA-DR expression was observed both in dermatoses with epidermal involvement (e.g. allergic contact dermatitis and lichen planus) and those without (e.g. granuloma annulare and erythema nodosum).

As the only constant finding, presence of HLA-DR on keratinocytes, was invariably linked to dermal lymphocytic infiltrates; lymphocytic exocytosis into the epidermis not being a prerequisite. The close linkage of HLA-DR presence on keratinocytes with a lymphocytic inflammatory infiltrate is illustrated by the results in vasculitic lesions (Fig. 2). All specimens of 'lymphocytic vasculitis' were HLA-DR positive whereas those of leukocytoclastic vasculitis, which is characterized by neutrophilic predominance, were all negative. However, in pseudolymphoma and erythema annulare centrifugum dermal lymphocytic infiltrates were not associated with HLA-DR expression on the overlying keratinocytes (Fig. 3b). Phenotypic characterization of the lymphocytic infiltrate by double labelling experiments revealed that activated T-lymphocytes (Leu 4+, HLA-DR+ and Leu 4+, TEC-MLR-3+) were a major constituent both in dermatoses which typically featured HLA-DR+ keratinocytes (Fig. 4) and in skin disorders which, despite an abundance of inflammatory cells, consistently lacked HLA-DR-bearing keratinocytes (i.e. pseudolymphoma and erythema annulare centrifugum) (Fig. 3).

## DISCUSSION

In this study we have added another 27 dermatoses to the growing list of skin diseases which are associated with HLA-DR expression on keratinocytes. Furthermore, our results confirm previous reports which point out the close relationship of HLA-DR+ keratinocytes with the presence of an underlying lymphocytic infiltrate (Aiba & Tagami, 1984; Lampert, 1984). However, in two dermatoses, erythema annulare centrifugum and pseudolymphoma, the characteristic dense lymphocytic infiltrate was not accompanied by the occurrence of HLA-DR+ keratinocytes. This discrepancy might be due to differences in the cellular composition of the inflammatory infiltrates, the decisive factor most probably being the presence of activated T cells (Nickoloff *et al.*, 1985); these cells are generally considered to be the source of IFN-$\gamma$ (Kirchner & Marcucci, 1984), a lymphokine which has been shown to be capable of inducing HLA-DR antigen on keratinocytes *in vitro* (Basham *et al.*, 1984; Auböck *et al.*, 1985). A number of dermatoses in which we found HLA-DR+ keratinocytes have recently been reported to contain activated T-lymphocytes in the dermal infiltrates (Buechner, Winkelmann & Banks, 1983, 1984; Ralfkiaer & Wantzin, 1984) Also, our double labelling experiments confirmed the presence of activated T cells (Leu 4+, HLA-DR+ or Leu 4+, TEC-MLR-3+) in skin disorders associated with HLA-DR+ keratinocytes. In view of this, however, it is surprising that comparable numbers of activated T-lymphocytes were also found in the dermal infiltrates of those dematoses in which HLA-DR+ keratinocytes were not found (i.e. erythema annulare centrifugum and pseudolymphoma). The presence of activated T-lymphocytes and IFN-$\gamma$ production by these cells may not be therefore the sole factor regulating HLA-DR induction in keratinocytes of diseased skin. Alternatively, activation of T cells as phenotypically defined may not necessarily imply concomitant functional activation, with synthesis and secretion of IFN-$\gamma$.

The functional role of 'aberrant' HLA-DR expression is still a matter of speculation. It could contribute to the induction (Bottazzo *et al.*, 1983), perpetuation (Bottazzo *et al.*, 1983) or down-regulation (Taramelli *et al.*, 1984) of immune responses, or be an epiphenomenon. Our results on keratinocytes of diseased skin do not favour an inductive role, as we never observed HLA-DR positive keratinocytes in the absence of, or preceding, a dermal infiltrate. This is consistent with a recent study showing that in the tuberculin reaction, HLA-DR positive keratinocytes emerge 2–4 days after the appearance of the dermal infiltrate (Scheynius & Tjernlund, 1984). The perpetuating effects of HLA-DR-positive keratinocytes may be speculated upon, for instance, in allergic contact dermatitis and infectious dermatoses as a result of their possible antigen presenting capacity. This possibility has recently been suggested also for HLA-DR+ thymocytes (Londei *et al.*, 1984) and melanoma cells (Guerry *et al.*, 1984).

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# An association between HLA DR1 and lichen planus

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## SUMMARY

Serological typing for HLA Class II antigens in 72 patients with lichen planus (LP) revealed a highly significant association with HLA DR1 and MT1 (DQw1). DR1 was present in 80% of patients with generalized LP, 54% with localized LP, 56% of patients with drug-induced LP and in 31% of patients with mucosal LP, compared with 25% of normal controls. MT1 (DQw1) was found in 83% of the LP group and 62% of the normal controls. These findings strongly suggest a genetic predisposition or susceptibility to the development of generalized LP and perhaps also to drug-induced LP.

Lichen planus (LP) is a clinically distinctive chronic papulosquamous skin disorder. An autoimmune aetiology of LP has been postulated (Black, 1977) and LP has been reported in patients with lupus erythematosus (Copeman, Schroeter & Kierland, 1970), myasthenia gravis (Tan & Samman, 1974a), alopecia areata and vitiligo (Tan & Samman, 1974b), bullous pemphigoid (Stingl & Holubar, 1975) and primary biliary cirrhosis (Powell, Rogers & Dickson, 1983). The presence of abnormal levels of serum immunoglobulin in some patients with LP (Stankler, 1975), the detection of immune reactants at the basement membrane zone using direct immunofluorescence techniques (Abell *et al.*, 1975), and the recent demonstration of circulating antibodies reactive with epidermal antigens in patients with LP (Olsen *et al.*, 1984), are all supportive evidence of an autoimmune pathogenesis. The recognition of the LP type eruption seen in chronic graft-versus-host disease after bone marrow transplantation (Saurat, *et al.*, 1975), and the occurrence of LP in other immunodeficiency states (Flamenbaum *et al.*, 1982) has renewed interest in the aetiology and pathogenesis of LP and also in a possible relationship to alteration in host immunity.

Familial LP has been recognized since 1884 (Saffron, 1940) and recent reports have confirmed this entity (Mahood, 1983). An early HLA study of patients with LP showed HLA-A3 to be significantly increased (Lowe, Cudworth & Woodrow, 1976). Subsequent studies although showing a trend towards an increase in HLA-A3 in LP patients, did not demonstrate statistically significant differences from controls (Saurat *et al.*, 1977; Veien *et al.*, 1979).

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Because some diseases have been shown to be more closely related to products of the D locus genes than A or B genes, and as host immune responses may be governed by genes located on the short arm of chromosome number 6 close to the HLA-D locus, we performed HLA-DR typing on patients with lichen planus.

MT antigens, which are Class II antigens closely related to DR, were also typed for during this study. MT antigens are generally regarded as supertypic to DR specificities since each MT specificity is strongly associated with a cluster of different DR antigens. As the study was begun prior to the 9th Internatonal Histocompatibility Workshop in 1984, the original antigen designations will be used in this paper. However, the current nomenclature for MT1 is DQw1, MT2 is DRw52, MT3 is DRw53 and MT4 is DQw3.

## METHODS

Seventy-two lichen planus patients seen in the Department of Dermatology of the Mayo Clinic over a 2-year period from 1982 to 1984 had HLA-DR and MT typing carried out. The diagnosis of LP was based on its typical clinical appearance and distribution, with additional histological confirmation in 48 patients. The lichen planus was classified clinically into three categories according to the distribution and extent of cutaneous involvement. Generalized LP patients ($n = 20$) had 20% or more cutaneous involvement with LP, localized LP patients($n = 39$) had less than 20% cutaneous involvement, and mucosal LP patients ($n = 13$) had oral and/or genital involvement without cutaneous lesions. Nine of the patients in this study were suspected of having drug induced LP (D-penicillamine 3, gold 2, diuretics 2, quinidine 1 and phenolphthalein 1).

HLA-DR and MT typing was carried out in the Mayo Clinic Blood Bank on peripheral blood lymphocytes. One hundred and seventy-one normal blood donors were studied during the same time period, using the same methods and antisera. A microdrop lymphocyte cytotoxicity method was employed, and testing was carried out in the laboratory without knowledge of the clinical status of the individuals. B lymphocytes for DR typing were prepared by separation of peripheral blood lymphocytes over nylon wool columns. Antigens tested were HLA-DR 1, 2, 3, 4, 5, 6y, 7, 8, 9, and MT 1, 2, 3, 4. Serum tray sets used for typing included sera supplied by Dr P. I. Terasaki, and also locally obtained sera further defined both in international cell exchanges and in the 8th International Histocompatibility Workshop (1980). DR 6y is a designation used to describe a complex of DR6 and serologically closely-related antigens, subsequently categorized as DRw13 and DRw14 in the 9th International Histocompatibility Workshop. The MT/DR associations are as follows:

| MT | DR |
|---|---|
| MT 1 | 1, 2, 10, 6y |
| MT 2 | 3, 5, 8, 6y |
| MT 3 | 4, 7, 9 |
| MT 4 | 4, 5, 8 |

Statistical analysis of the results was carried out and a two-tail *P*-value calculated using a one degree of freedom chi square test of associaton or Fisher's exact test as appropriate. The relative risk (RR) for each DR and MT antigen was calculated using the formula:

$$\frac{PD/(1\text{-}PD)}{PC/(1\text{-}PC)}$$

where PD is the proportion of LP patients with each antigen, and PC is the corresponding proportion in the controls. The population attributable risk $\sigma$ was calculated and defined as follows (*see* Bengtssan & Thomson, 1981):

$$\sigma = \frac{\text{PD-PC}}{1\text{-PC}}$$

## RESULTS

There were 38 females and 34 males in the group of 72 lichen planus patients. Thirty-nine patients had localized LP, 20 had generalized LP and 13 had mucosal involvement alone. The age of onset of LP ranged from 21 to 84 years, with a mean age of onset of 44·6 years. The follow-up from date of diagnosis ranged from 1 month to 43 years, with a mean follow-up of 92 months.

The results of HLA-DR and MT typing are summarized in Table 1 together with the calculated corrected $P$ values, relative risk (RR) and the population attributable risk ($\sigma$). HLA-DR1 and MT1 were found to be significantly increased in LP patients, with $P$ values of $<0{\cdot}00013$ and $<0{\cdot}01417$, respectively. The relative risk for patients with DR1 of developing LP was 3·94 while the population attributable risk was 0·42. The comparable figures for MT1 were 3·07 (RR) and 0·56 ($\sigma$). Five of the nine patients with drug induced LP (56%) had DR1. DR1 was most frequent in patients with generalized induced LP occurring in 16 patients (80%) and was least frequent in those with mucosal LP (31%). Fifty-four per cent of patients with localized LP had HLA-DR1 (Table 2).

TABLE 1. Results of HLA-DR and MT typing in lichen planus patients and controls

| | Lichen Planus ($n=72$) | | | Control ($n=171$) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Antigen | Present | % | Absent | Present | % | Absent | Corrected$^{(1)}$ $P$ | RR$^{(2)}$ | $\sigma^{(3)}$ |
| DR1 | 41 | 56·9 | 31 | 43 | 25·2 | 128 | <0·00013 | 3·94 | 0·42 |
| DR2 | 12 | 16·7 | 60 | 45 | 26·3 | 126 | 1·3654 | 0 56 | −0·13 |
| DR3 | 16 | 22·2 | 56 | 37 | 21 6 | 134 | 11·9562 | 1·03 | 0 01 |
| DR4 | 16 | 22·2 | 56 | 48 | 28·1 | 123 | 4·4802 | 0·73 | −0·08 |
| DR5 | 12 | 16·7 | 60 | 43 | 25·2 | 128 | 1 9369 | 0·60 | −0·11 |
| DR6y | 13 | 18·1 | 59 | 37 | 21·6 | 134 | 6·8673 | 0 80 | −0 05 |
| DR7 | 10 | 13·9 | 62 | 35 | 20·5 | 136 | 2 9627 | 0·63 | −0 08 |
| DR8 | 4 | 5 6 | 68 | 8 | 4 7 | 163 | 9 7891* | 1·20 | 0 01 |
| DR9 | 4 | 5·6 | 68 | 5 | 2·9 | 166 | 5·9325* | 1·95 | 0 03 |
| MT1 | 60 | 83 3 | 12 | 106 | 62 0 | 65 | 0·01417 | 3·07 | 0 56 |
| MT2 | 44 | 61·1 | 28 | 114 | 66 7 | 57 | 5·29087 | 0·79 | −0·17 |
| MT3 | 24 | 33·3 | 48 | 86 | 50·3 | 85 | 0·1989 | 0·49 | −0 34 |
| MT4 | 30 | 41 7 | 42 | 80 | 46·8 | 91 | 6 03629 | 0 81 | −0 10 |

(1) Two-tail $P$-value from a one-degree of freedom chi-square test of association except where noted. Corrected for numbers of antigens tested.

(2) RR refers to the relative risk as defined in Methods.

(3) Population attributable risk (*see* Methods).

* Two-tail $P$-value from Fisher's Exact Test.

TABLE 2. Percentage of patients DR1-positive according to type of lichen planus

| | No. of patients | % DR1 |
|---|---|---|
| Localized LP | 39 | 54 |
| Generalized LP | 20 | 80 |
| Mucosal LP | 13 | 31 |
| Drug-induced LP | 9 | 56 |

## DISCUSSION

Our study shows a striking statistically significant increase in the frequency of HLA-DR1 in LP patients. The increased frequency of DR1 in patients with localized, generalized and drug-induced LP is supportive of a common disease entity. The relative low frequency of DR1 in patients who only have mucosal LP may indicate that this group of patients includes more than one genetic or pathogenetic entity, even though they present with clinical and histological findings suggestive of LP.

HLA-A3, which was increased in some previous HLA studies of patients with LP is known to be in linkage disequilibrium with DR1 (Batchelor, 1980). Thus, a moderately increased frequency of HLA-A3 may in fact have been reflecting a stronger association with the Class II antigen HLA-DR1. In 94% of individuals with DR1 antigen, MT1 or MB1 will also be detected, i.e. DR1 is 'included' in MT1 (Duquesnoy & Marrari, 1980). Therefore, the finding of a statistically significant association between MT1 and LP may be a secondary phenomenon.

The relationship of HLA antigens to clinical disease is speculative. They may function as cell surface receptors for drugs or infectious agents (Helenius *et al.*, 1978), or share antigenic determinants with infectious agents which allow the latter to escape the host immune response through tolerance or molecular mimicry (Svejgaard *et al.*, 1975). DR1 antigen has been associated with reduced serological response to Epstein Barr viral capsid antigen (Cohen *et al.*, 1984), and MT3 has been shown to be involved in the presentation of mumps viral antigen to T cells (Ball & Stastny, 1984) suggesting that several different Class II antigen allelic systems, including MT and DR, may be involved in the induction phase of the immune response to alloantigens. Immune response genes are thought to be intimately linked to the DR locus, and are expressed through Ia antigens on T-lymphocytes, macrophages, endothelial cells and the Langerhans cells of the epidermis. Some of these Ia antigen-carrying cells are involved in LP. In early LP epidermal Langerhans cells are increased, even before the arrival of the characteristic mononuclear cell infiltrate (Ragaz & Ackerman, 1981). The cellular infiltrate in LP has been shown to consist almost entirely of T-lymphocytes mainly of the helper subset (McMillan *et al.*, 1981; Gomes *et al.*, 1982).

It is possible that, in certain genetically-predisposed individuals with specific immune response genes, Langerhans cells may process foreign antigens in an abnormal fashion and, in doing so, liberate soluble monokines which in turn mediate a local lymphocytic response. This lymphocytic infiltrate would be directed not only against Langerhans cells but also against other epidermal cells, some of which are known to express Ia antigen in both LP and graft-versus-host disease. This sequence of events could be initiated by infectious agents, by contact with gold or colour photograph developers or systemic medication, many of which are deposited in the skin.

The presence of LP in patients with disorders of immune reactivity would be consistent with this postulated type of altered antigen processing.

Our study has shown that LP is significantly associated with DR1 and MT1. Since the association between LP and DR1 is not 100%, it may be that DR1 is simply in strong linkage disequilibrium with the true LP disease gene. Further studies of familial cases of LP are now needed to clarify the genetics of this disease.

## ACKNOWLEDGMENTS

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# Occupational exposure to ultraviolet radiation in dermatology departments

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## SUMMARY

The ultraviolet radiation (UVR) doses received by staff who are occupationally exposed to artificial sources of UVR found in dermatology departments have been measured using personal UV monitors. The results show that staff may receive actinic (UVB and UVC) doses which are in excess of recommended maximum permissible limits for occupational exposure, but that the UVA doses are well within these limits.

Ultraviolet radiation (UVR) is central to the management of many dermatological diseases. In the Swedish city of Gothenburg (population approximately 420 000) more than 40 000 exposures of therapeutic UVR are given annually. The most common modality is UVB irradiation but psoralen photochemotherapy (PUVA) is also widely used, and treatment is given either in hospital or in specially-equipped day care centres (Larkö, 1982). We have discussed previously the long-term safety of phototherapy (Larkö & Swanbeck, 1982), and have compared the therapeutic ultraviolet exposure of patients with natural exposure occurring in different outdoor leisure pursuits (Diffey, Larkö & Swanbeck, 1982) and in different occupations (Larkö & Diffey, 1983).

There are no published data on the levels of UVR received by staff engaged in phototherapy, even though standards regarding occupational exposure to UVR have been in existence for a decade. The purpose of the present study was to measure the UVB and UVA received on the lapel site by workers employed principally in the UVR environment of dermatology departments, and by control groups working in non-radiation areas.

At present there are no internationally agreed limits for occupational exposure to UVR. The most comprehensive occupational exposure standard for incoherent (non-laser) UV sources which has been published is that of the American Conference of Governmental Industrial Hygienists. This standard has been endorsed by the National Institute for Occupational Safety and Health (NIOSH, 1972) in the United States and adopted as a voluntary standard by many

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TABLE 1. Subjects monitored for ultraviolet radiation exposure at each workplace and the type of phototherapy equipment in use

| Workplace | No. subjects monitored | No. pairs of film badges | | Phototherapy equipment | | |
|---|---|---|---|---|---|---|
| | | Issued | Returned | No. units | Type of unit | Number and type UV lamp |
| Phototherapy I | 10 | 200 | 115 | 8 | UVB whole body 'upright' cabin | 14 Philips TL40W/12<br>14 Philips TL20W/12 |
| | | | | 1 | UVB hand and foot | 6 Philips TL20W/12 |
| | | | | 2 | PUVA 'upright' cabin | 22 Philips TL85W/09 |
| | | | | 2 | PUVA hand and foot | 14 Sylvania F8T5/PUVA<br>12 Sylvania F15T8 |
| Phototherapy II | 10 | 200 | 160 | 2 | UVB whole body 'upright' cabin | 14 Philips TL40W/12<br>14 Philips TL20W/12 |
| | | | | 1 | UVB 'lie-down' unit | 14 Sylvania F75/85W/UV21 |
| | | | | 2 | UVB hand and foot | 6 Philips TL20W/12 |
| | | | | 1 | UVB/A 'lie-down' unit | 20 Wolff Helarium |
| | | | | 1 | PUVA 'upright' cabin | 22 Osram L100W/79 |
| | | | | 1 | PUVA 'upright' cabin | 22 Airam 80W |
| | | | | 2 | PUVA 'lie-down' unit | 40 Sylvania FR90T12/PUVA/HO |
| | | | | 1 | UVASUN 2000 | filtered metal halide lamp |
| | | | | 5 | PUVA hand and foot | 14 Sylvania F8T5/PUVA<br>12 Sylvania F15T8 |
| General dermatology | 5 | 100 | 79 | 2 | UVB whole body 'upright' cabin | 11 Philips TL40W/12 |
| | | | | 1 | PUVA 'upright' cabin | 22 Philips TL85W/09 |
| Outpatient department | 10 | 200 | 194 | | No phototherapy equipment | |
| Ward | 10 | 200 | 144 | | No phototherapy equipment | |

European regulatory authorities, including those in the U.K. and Sweden. Separate exposure standards are given for UVA, and for the actinic UVB and UVC wavelengths.

For the spectral region 400–315 nm (UVA), the total irradiance incident on unprotected eyes and skin for periods of greater than 1000s should not exceed 10 W/m$^2$, and for exposure times of 1000 s or less the total radiant exposure of unprotected eyes and skins should not exceed 10$^4$ J/m$^2$.

The exposure standard for the spectral region 315–200 nm (UVB and UVC) is based on an envelope action spectrum which combines the photokeratitis and skin erythema action spectra and which is defined as a smooth curve somewhat below the energies required for the development of observable effects (Sliney, 1972). The standard, which applies to occupational exposure during an 8 h working day, shows maximum sensitivity at a wavelength of 270 nm and recommends a maximum permissible exposure (MPE) dose of 30 J/m$^2$.

## METHODS

### *Subjects*

A total of 45 subjects took part in the study. Each subject worked principally in one of the five different situations listed in Table 1 and wore two film badges (one for recording actinic (UVB and UVC) radiation and one for recording UVA) on the lapel during working hours (including rest periods). Although it is the face and the backs of the hands that are most at risk, measurements of the anatomical distribution of sunlight falling on both a manikin and on living subjects have shown that the dose received on the lapel site gives a reasonable approximation of that received by the face and hands (Diffey, 1985). The film badges were changed twice weekly for 10 weeks and the exposure time for which each pair of film badges had been worn was noted. The range of times for which a given film badge was worn varied from 5 to 48 h with a median time of between 20 and 30 h at each of the five workplaces. The study was carried out during the winter of 1984/85 to minimize the influence of natural UVR, particularly UVB, on the recorded doses.

### *Ultraviolet radiation sources*

A variety of sources are used for both UVB phototherapy and PUVA photochemotherapy (Table 1). In nearly every case the lamps are of the fluorescent type incorporating phosphors designed to emit UVR mainly in the UVB or UVA regions (Diffey, 1982).

### *Ultraviolet radiation dosimeters*

Personal exposure to actinic UVR was recorded using the polymer film, polysulphone, 40 $\mu$m thick (Davis, Deane & Diffey, 1976). The basis of the method is that when polysulphone film is exposed to UVR at wavelengths less than 330 nm, the UV absorption of the film increases. The increase in absorbance measured at 330 nm ($\Delta A_{330}$) increases with UV dose, and so the film readily lends itself to application as a UV dosimeter (Diffey, 1984). Exposure to UVA was measured in a similar way by using a polyvinyl chloride (PVC) film incorporating 8-methoxypsoralen (8-MOP) as the chromophore (Diffey & Davis, 1978). In this instance the UV absorption of the film measured at 305 nm decreases with increasing UV dose.

The polysulphone film badges were calibrated using a Philips TL20W/12 lamp; this lamp is the sort most commonly used for UVB phototherapy. The actinic irradiance was measured using an International Light model 730A actinic radiometer; this radiometer has a spectral response closely matching the 'hazard action spectrum' for occupational exposure to actinic

UVR and displays an effective irradiance in W/m² relative to 270 nm (Diffey, 1982). The actinic radiometer was calibrated using a deuterium discharge lamp which had recently been calibrated by the National Physical Laboratory. The actinically-effective UVR dose ($UVR_{actinic}$) was shown to be related to the increase in optical absorbance at 330 nm ($\Delta A_{330}$) by the expression:

$$UVR_{actinic} = 7 \times 10^3 (\Delta A_{330})^{1\ 38} \quad J/m^2 \tag{1}$$

Calibration of the 8-MOP film badges was carried out with a Philips TL20W/09 fluorescent lamp, which emits mainly UVA radiation. The UVA irradiance was measured with a 'Uvichek' UVA meter which was calibrated using the method described by Stobbart & Diffey (1980). The UVA dose was found to be related to the decrease in optical absorbance of the 8-MOP films at 305 nm ($\Delta A_{305}$) by the expression:

$$UVA = 1{\cdot}5 \times 10^5 \ (\Delta A_{305})^{1\ 38} \quad J/m^2 \tag{2}$$

The 8-MOP film also reacts to the radiation from UVB phototherapy lamps. However, measurements showed that for an actinically-effective UVR dose of 30 J/m² (i.e., the maximum permissible exposure for an 8 h period) from a Philips TL20W/12 lamp, the change in optical absorbance at 305 nm of an 8-MOP film was 0·009, equivalent to a 'UVA exposure' of only 0·1% of the UVA MPE for the same period.

After being worn by the participants in the study, the film badges were returned to one of the authors (BLD) for spectrophotometric assay and calculation of UV dose. In a few cases, the film badges were damaged and determination of the UV dose was not possible.

## RESULTS

### *Actinic UV Doses*

A summary of the actinic UV doses recorded by the polysulphone film badges is given in Table 2. In calculating the 8 h actinic dose for a given polysulphone film badge, the UV dose estimated

TABLE 2. The 8 h actinic ultraviolet doses recorded by polysulphone film badges worn in five different workplaces

| | Number of film badges | | | | |
|---|---|---|---|---|---|
| 8 h actinic UV dose (J/m²) | Phototherapy I | Phototherapy II | General dermatology | Outpatient department | Ward |
| < 1 | 23 | 14 | 30 | 38 | 49 |
| 1–3 | 21 | 18 | 28 | 35 | 34 |
| 3–10 | 43 | 61 | 15 | 83 | 48 |
| 10–30 | 19 | 38 | 5 | 33 | 9 |
| 30–100 | 8 | 24 | 1 | 5 | 1 |
| 100–300 | 0 | 5 | 0 | 0 | 0 |
| > 300 | 0 | 0 | 0 | 0 | 0 |
| Median dose (J/m²) | 5 2 | 8 1 | 1 3 | 4 0 | 2 4 |
| % film badges exceeding MPE (30 J/m²) | 7·0 | 18 1 | 1 3 | 2 6 | 0 7 |

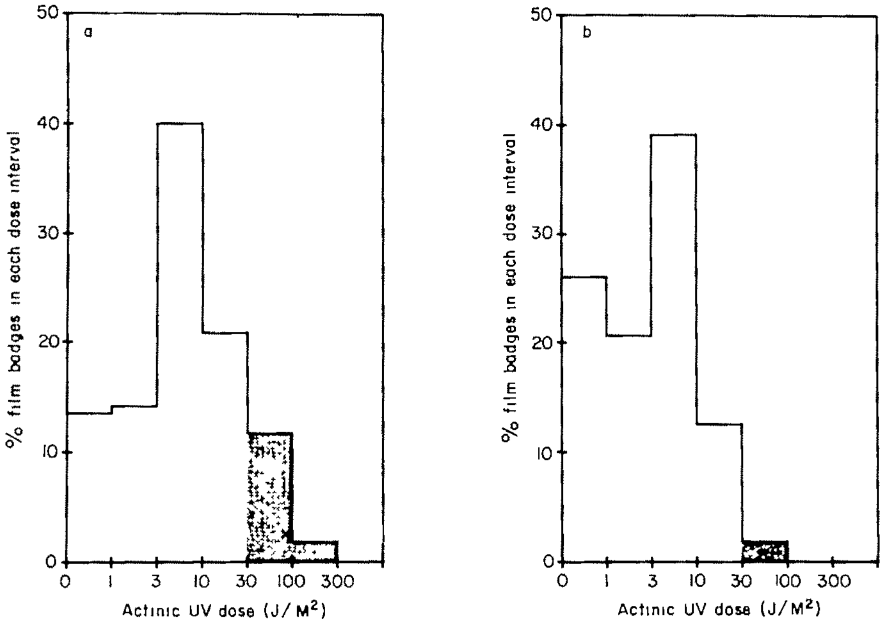

FIGURE 1. Actinic UV doses recorded in (a) Phototherapy areas ('Phototherapy I' and 'Phototherapy II') and (b) Control areas ('Outpatient Department' and 'Ward'). The shaded areas indicate those film badges which received doses in excess of the 8 h maximum permissible exposure limit of 30 J/m².

by equation 1 was multiplied by a factor of 8/h, where h was the number of hours for which the film badge had been worn. A chi-squared test showed that the doses recorded at the five workplaces were significantly different ($P < 0{\cdot}001$). Not surprisingly, the highest doses were received by those working environments where UVR sources were employed. In 'Phototherapy II' nearly one fifth of the film badges recorded an 8 h actinic dose in excess of the maximum permissible exposure of 30 J/m². A few film badges worn in the non-radiation areas (Outpatients and Ward) received unexpectedly high UV doses, attributable to occasional visits by nurses to 'UV areas' of the department, or to surface damage or contamination of the film badges resulting in an abnormally high absorbance and hence a falsely high calculated dose. Figure 1 illustrates the tendency for staff employed in the phototherapy areas to receive higher doses than those in the control areas (Outpatients and Ward).

### *UVA Doses*

The UVA exposures recorded by the 8-MOP film badges were well within recommended occupational exposure limits. The median UVA exposures in all five workplaces were 0·1% or less of the maximum permissible exposure and in no case did an 8-MOP film badge receive a dose exceeding the MPE.

## DISCUSSION

Our data have shown clearly that people working with phototherapy equipment are potentially at risk from exposure to ultraviolet radiation. The UVA exposures were well within recommended occupational exposure limits but the doses of actinic UVR received in some instances give some cause for concern. One reason why a larger proportion of the film badges worn in 'Phototherapy II' recorded actinic doses in excess of the MPE than those worn in 'Phototherapy I' was that during the period of the study many patient exposures to UVB radiation for research purposes were administrated by staff in the former working area. We believe that the data recorded in 'Phototherapy I' are more representative of typical working practices. The highest dose recorded during the study was 120 J/m$^2$ and even this is probably too low to produce delayed erythema in normal Caucasian skin over an exposure period of 8 h.

Recommendations on occupational exposure to artificial sources of ultraviolet radiation stress that exposure of skin and eyes should be kept to a minimum in view of the potential long term carcinogenic and cataractogenic risks of repeated UVR exposure (WHO, 1979). Measures which can be taken to minimize the unnecessary exposure of staff to UVR include proper engineering design of UVR-emitting installations and suitable enclosures, coating of reflecting surfaces, such as walls and panels, with a paint of low UV reflectance, wearing of appropriate goggles and/or face shields accompanied, if necessary, by suitable UVR-opaque clothing, limiting access to persons directly concerned with the work, and finally, ensuring that staff are aware of the potential hazards associated with exposure to ultraviolet radiation sources.

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**Pharmacology and Treatment**

# A new device for the treatment of hyperhidrosis by iontophoresis

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## SUMMARY

A new device for the treatment of hyperhidrosis by iontophoresis is described. Twenty-five patients have so far been treated, six with hyperhidrosis of the palms, 13 with hyperhidrosis of the soles and six with axillary hyperhidrosis. In 21 cases there was an excellent result. The effect of the treatment usually lasted for several weeks. Maintenance treatment every 4–6 weeks was found to be required.

The medical treatment of idiopathic hyperhidrosis is usually ineffective in all but the mildest cases. Surgical treatment includes sympathectomy for hyperhidrosis of the palms, with several potential complications, and excision of the sweat glands for axillary hyperhidrosis.

Shelley *et al.* (1948) described the effect of iontophoresis on sweat-glands. Tap water iontophoresis for treatment of hyperhidrosis was described by Boumann and Grunewald-Lentzer (1952) and Levit (1968). The reason suspected for the effect of iontophoresis was that the epidermis receives a nonspecific injury which results in abnormal keratinization and hyperkeratotic plugging of the sweat-gland orifices. Ordinary tap water served well and treatments were given at two- to three-day intervals, usually over one or two weeks. The decrease in sweating was variable and lasted for up to three months.

Iontophoresis with tap water or anticholinergic compounds (poldine methosulphate or glycopyrronium bromide) can be very effective for treating palmar and plantar hyperhidrosis (Abell & Morgan, 1974; Grice, Sattar & Baker, 1972), but the treatment of axillary hyperhidrosis has been less successful.

Recently, a new electric transformer has been constructed by means of which it is possible also to treat axillary hyperhidrosis successfully. We report the results obtained using this device.

## METHODS

The new iontophoresis machine is a self-regulating, constant-voltage, constant-current transformer (LB Trading Aps, Copenhagen, Denmark), as used in industrial galvanization. The output is a constant direct current with an overlying pulsating direct current. The maximum voltage is 50 volts and the maximum current 20 milliamps, which minimizes the possibility of side effects.

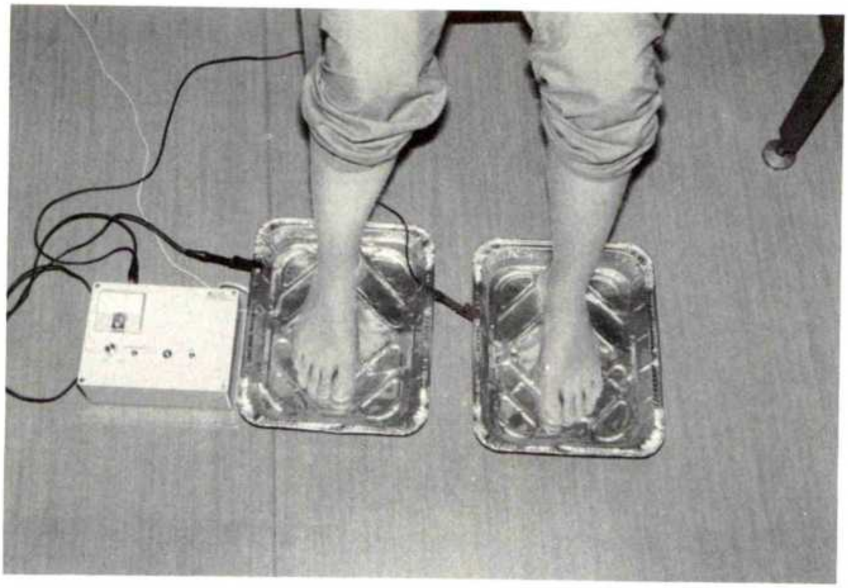

FIGURE 1. Arrangement for treatment of the feet with iontophoresis.

To administer the treatment, the hands or feet were placed in a shallow aluminium pan containing enough tap water to cover the palms or soles, breaks in the skin surface having first been covered with vaseline. The pans were connected to the machine which was switched on at minimum current. The current was slowly increased until the patient felt some discomfort, and was then slightly reduced and permitted to flow for 5–10 min. At the end of this period the current was slowly reduced to zero, and the patient then removed his hands or feet from the

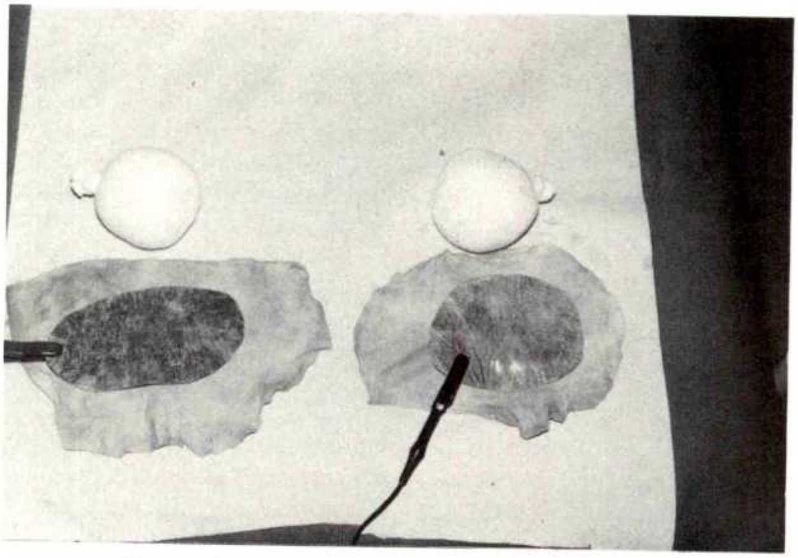

FIGURE 2. Details of the electrodes used to treat the axillae with iontophoresis.

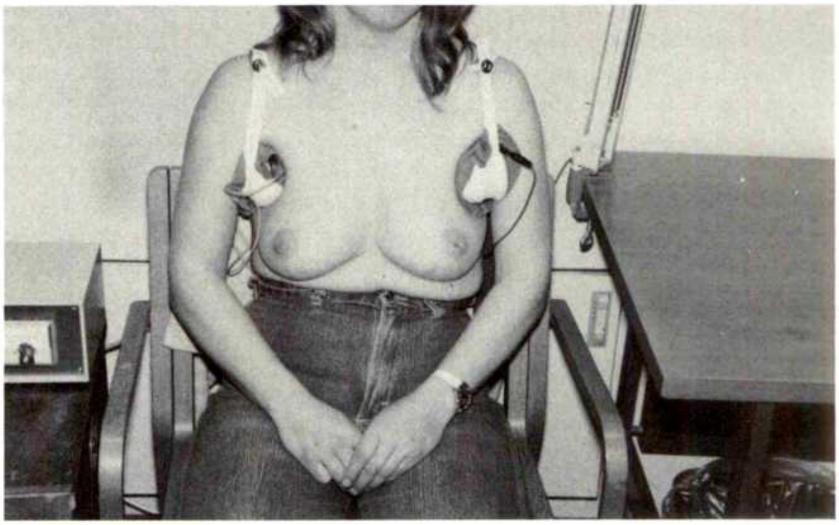

FIGURE 3. Arrangement for treating the axillae with iontophoresis.

water. The polarity was then reversed, the patient re-inserted his hands or feet and the current was again raised for a second period of 5–10 min. In this way each side was subjected to the action of the anodal current for half of the total treatment time. The current was then slowly reduced to zero and the patient removed his hands or feet from the water. The current was always raised or lowered slowly to avoid discomfort. Treatment of the feet is shown in Figure 1.

A special electrode has been constructed to treat axillary hyperhidrosis. It consists of a 10 × 8 cm lead plate, 1 mm thick. The plate is bent over a ball of tubular gauze stuffed with cottonwool. The lead plate is covered with a wet wash-leather and connected to the iontophoresis apparatus by means of a crocodile clip. The details are shown in Figure 2. To ensure a good contact with the top of the axillae the electrodes are held in place by a rubber strap as shown in Figure 3. Initially, the patients were treated 2–3 times weekly. When a sufficient effect was achieved the intervals were gradually increased. The average interval between maintainence treatments was 4 weeks.

## RESULTS

Twenty-five patients have been treated, six with hyperhidrosis of the palms, 13 with hyperhidrosis of the soles and six with axillary hyperhidrosis. In 21 cases the treatment was entirely effective, in 3 cases there was an improvement but the treatment was not completely effective and in one case of axillary hyperhidrosis the effect was doubtful.

During follow-up, most of the patients have been kept free from hyperhidrosis for up to a year by a treatment every 4–6 weeks. To illustrate our results two cases are reported in detail.

### *Case 1: Patient with hyperhidrosis of the feet*

A 40-year-old woman had suffered since puberty from severe hyperhidrosis of the feet. She always had to change her socks four to five times a day and always had problems with her shoes which were usually ruined after one or two weeks by her excessive sweating. All local treatment had been ineffective.

Iontophoretic treatment of the patient's feet was started three times weekly. There was no effect after the first three treatments but after six treatments the results were excellent. Her feet now remained completely dry and the strong odour disappeared. The patient also found that she could buy a smaller size of shoe. She has been kept free from sweating of the feet for approximately a year by treatment every 4–6 weeks.

### *Case 2: Patient with axillary hyperhidrosis*

The patient was a 24-year-old woman who had for many years suffered from severe hyperhidrosis of the axillae and the palms. All treatment, local and systemic, had been ineffective. The copious axillary sweating had rapidly ruined her clothing. She also suffered from an embarrassing odour of which she was constantly aware. She always had to change her blouse two to three times a day. For many years she had also suffered from wet hands which bothered her, especially when she was knitting, when she would have to wash her hands every 15 to 20 min.

After only one treatment with iontophoresis of the palms, the patient experienced, for the first time in many years, a day with dry hands. After four treatments the axillae also were completely dry. Both axillae and palms are, at present, kept dry by treatment every 4 weeks.

## DISCUSSION

There have been several papers on the treatment of hyperhidrosis with iontophoresis (Boumann & Grunewald-Lenzer, 1952; Grice *et al.*, 1972; Levit, 1980; Morgan, 1980). All the authors agree that the effect of this treatment is excellent and our results support this. Especially, we found the treatment to be so effective in axillary hyperhidrosis that we would recommend iontophoresis in preference to surgical excision of the sweat-glands. However, the treatment has not been generally accepted and is not mentioned in the newer dermatology text books. One reason for this could be lack of suitable, readily available apparatus. The only other commercially available device seems to be that from the R.A.Fischer Company in California, U.S.A.

Although iontophoresis is agreed to be effective in the treatment of hyperhidrosis, its mode of action remains unknown. We have found that iontophoresis of the feet has an excellent effect on pustulosis. We suggest, therefore, that the mode of action is a local electro-chemical coagulation of proteins in areas where the current can flow easily because of a high level of electrolytes. This theory could explain why the effect of iontophoresis can last for several weeks, since this is the time it would take for damaged cells to be replaced.

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# Carbon dioxide laser vaporization of plaque psoriasis

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## SUMMARY

The treatment of circumscribed plaque psoriasis with a carbon dioxide ($CO_2$) laser as an out-patient procedure is reported in three patients. The growth of new tissue over the healed laser vaporized area was similar to normal skin and has remained free of psoriasis during a follow-up of 3·5 years. Laser vaporization appears to be a simple and effective alternative to conventional therapy in cases of plaque psoriasis.

A carbon dioxide laser is routinely used in our department for conization of the uterine cervix in cases of non-invasive neoplasia, and for treating skin disorders of the vulva such as kraurosis or condylomatosis.

Three staff members with circumscribed cutaneous plaque psoriasis gave informed consent for $CO_2$ laser vaporization treatment.

## METHODS

A high energy carbon dioxide laser, wavelength 10 600 nm (Sharplan 733) with continuous mode selection, was used. The laser arm was hand held and a defocused beam was used giving a spot size of 2 mm and a power density of 160–200 watts/cm$^2$ (power output 5–6 watts). With this procedure, the skin was destroyed to a depth of approximately 1 mm.

## CASE REPORTS

### *Case 1*

A 41-year-old woman, with an 8-year history of localized psoriasis, had previously been treated with topical steroids, tar and UV radiation with only a transient effect. In December 1981 she had intractable lesions on her elbows and forearms measuring 18 × 9 cm on the left arm and

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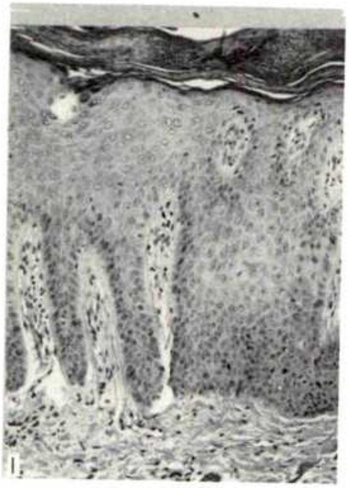
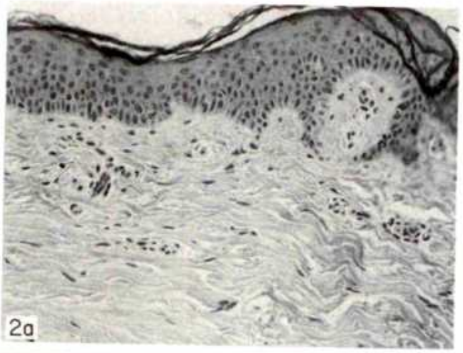
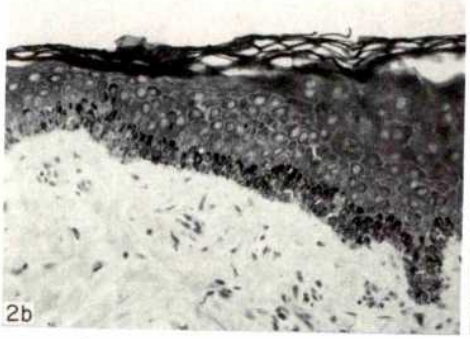
15 × 8 cm on the right. She had laser vaporization, under local anaesthesia, of a 20 × 20 mm area of the large plaque on her right elbow. The treated area healed in three weeks. In February 1982, a 60 × 25 mm area of the plaque on her left elbow was similarly treated; healing was uneventful and took 6 weeks. In March 1982, a 60 × 40 mm area at the edge of the large plaque on her right elbow and adjacent to the previously healed area was treated. Again, healing took 6 weeks, despite secondary wound infection requiring antibiotic treatment. Then, the entire area of psoriasis on both arms disappeared, including the untreated areas adjacent to those exposed to laser vaporization. The treated areas have remained free from psoriasis, although new plaques appeared on untreated skin on both elbows in October 1984, and a further course of laser vaporization treatment was given in June 1985.

After each treatment, the patient was able to return to work immediately, and healing was uneventful, without bleeding, pain, necrosis, or the Koebner phenomenon. The scar tissue was as soft and elastic as normal skin, with no loss of hair, and with only slight depigmentation.







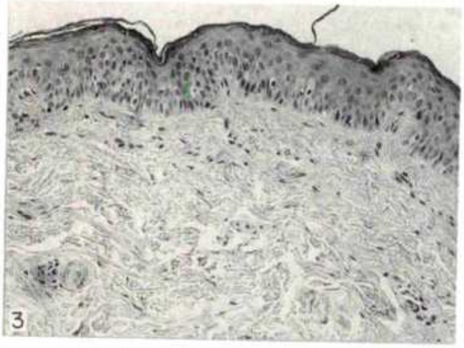


FIGURE 1. Surgical biopsy of plaque psoriasis before laser vaporization. (Case 1). Haematoxylin and eosin (original × 160). Typical psoriatic histology with parakeratotic and acantrotic epidermis; there is pronounced thinning over the elongated and club-shaped dermal papillae but no micro-abscesses.

FIGURE 2. Surgical biopsy of scar tissue from site of psoriatic plaque on left forearm two years after laser vaporization (Case 1). (a) Normal histology with no features of psoriasis (H & E; original × 160); (b) altered distribution of melanin pigment (Masson's stain; original × 240).

FIGURE 3. Surgical biopsy of clinically normal skin from the right forearm (Case 1). (H & E; original × 160).

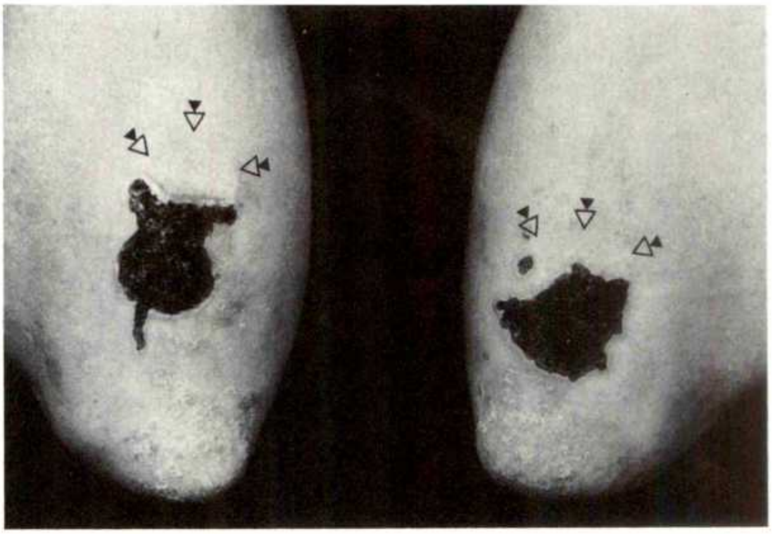


FIGURE 4. Appearance of plaques in Case 2 after treatment. Dark areas are the lesions caused by laser vaporization. Arrows show the healed area treated eighteen months earlier and showing depigmentation. Untreated psoriatic lesions can be seen on both elbows.

Biopsies from the psoriatic plaque on the left forearm before treatment (Fig. 1), from the same site 2·5 years after laser vaporization (Figs 2a and 2b), and from the corresponding uninvolved site on the right forearm (Fig. 3), showed the new tissue to be similar histologically to normal skin.

### *Case 2*

A 37-year-old woman with a 22-year history of plaque psoriasis on the limbs and scalp had been treated with courses of oral and topical steroids, dithranol, tar and UV radiation with little beneficial effect. The plaques always returned within 6 months of finishing treatment.

In March 1982, when plaques on her elbows and forearms had been present for over 3 years and measured 15 × 8 cm on the right arm and 16 × 7 cm on the left, she had laser vaporizaton treatment of an area of 35 × 35 mm on each arm. She had two further treatments to areas of 70 × 70 mm during the following 2 months. Although wound infection of the larger treated areas required systemic antibiotic treatment, healing was complete within 6 weeks on both occasions. Each section of the plaque was treated once only, until the whole plaque had been treated. Figure 4 shows freshly treated plaques.

There has been no recurrence of psoriasis on the patient's elbows and forearms 3 years after the first treatment and more than 1 year after the last treatment.

### *Case 3*

A 41-year-old man with a 12-month history of multiple large plaques on his forearms, knees and legs had previously been treated unsuccessfully with topical steroids and UV radiation. A preliminary diagnosis of psoriasis was confirmed by histological examination prior to laser vaporization treatment. In a series of seven treatments, at 3, 6, or 12 week intervals between February 1984 and January 1985, each lesion was subjected to laser vaporization under local anaesthesia. A maximum area of 10 $cm^2$ was treated on each occasion. The patient was able to return to work immediately after treatment, all treated psoriatic plaques healed within 6 weeks without infection or pain, and there has been no recurrence of psoriasis 8 months after the last treatment.

## DISCUSSION

We report here laser vaporization treatment of circumscribed plaque psoriasis which had not responded to conventional therapy. To the best of our knowledge, this treatment has not previously been used in psoriasis (Békássy & Åstedt, 1984).

A series of treatments was given under local anaesthesia, each area being treated with vaporization once only. The maximum area that could be treated on a single occasion without subsequent complications seemed to be approximately 10 cm$^2$, since wound infection occurred in two of our patients when the treated areas exceeded 30 × 30 mm, whereas no infection occurred when this area was limited to 10 cm$^2$. However, in all cases healing was complete within 6 weeks, after which further laser treatment could be given. There was no bleeding, pain, necrosis, or Koebner phenomenon, and patients were able to return to work immediately after treatment.

Laser vaporization of psoriatic plaques destroys the lesion which is gradually replaced by scar tissue. The healed areas, although slightly depigmented, were as soft and elastic as normal skin, and hair growth was unimpaired. Histological examination showed that the new skin was normal in structure, apart from slightly altered pigmentation.

The laser vaporization seemed to have had a beneficial effect on adjacent untreated areas as observed in Case 1, where the whole plaque disappeared after the second treatment. However, this was transient as the psoriasis recurred in the untreated area two and a half years later. There has been no recurrence of psoriasis in any of the treated areas during the follow-up period, which in Case 1 is now 3·5 years.

The method described here causes destruction of psoriatic tissue which then heals completely. Colver, Cherry and Ryan (1984) using a Bio-las D helium-neon, a low-dose laser, irradiated psoriatic plaques without benefit, but their purpose was different from ours, being concerned with the inhibition of angiogenesis. If, as suggested by Telner and Fekete (1961), surface microvessels must be obliterated for psoriatic lesions to heal, the surface tissue destruction resulting from our approach should satisfy this criterion.

Randomized clinical studies are now needed to establish the value of this technique in the treatment of patients with plaque psoriasis.

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# Topical azelaic acid and the treatment of acne: a clinical and laboratory comparison with oral tetracycline

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## SUMMARY

Topical azelaic acid and oral tetracycline were compared in a 6-month double-blind study for treatment of acne vulgaris in 45 male subjects with clinical acne. Their acne was graded, inflamed or non-inflamed, lesions were counted and the density of their skin microflora was measured. Both treatments were of benefit and produced only a few minor side-effects. Although oral tetracycline was more effective than azelaic acid, the differences were only just significant. The average reduction in numbers of cutaneous micrococcaceae and *Propionibacterium sp.* with azelaic acid treatment was 224 and 30-fold, respectively.

In a separate group of 11 male subjects with physiological acne the effect of azelaic acid on sebum excretion rate was assessed, and little change was detected.

The two most commonly used topical preparations for acne in the U.K. are benzoyl peroxide and retinoic acid. Both are effective (Cunliffe *et al.*, 1981; Mills, Marples & Kligman, 1972) but both have disadvantages. They often produce a primary irritant dermatitis (Cunliffe & Burke, 1982) and benzoyl peroxide bleaches hair and clothes.

Topical antibiotics have been used in the U.S.A. for acne (Fulton & Pablo, 1974; Thomsen *et al.*, 1980; Taaffe *et al.*, 1981), but may induce antibiotic resistance (Eady, Holland & Cunliffe, 1982a).

There remains, therefore, a need for an effective topical therapy with none of these problems. Nazzaro-Porro *et al.* (1983) showed, in an open study with 100 patients, that azelaic acid, a naturally occurring dicarboxylic acid, was effective in acne. The purpose of our investigation was to compare topical azelaic acid and oral tetracycline, a common treatment for acne, in a double-blind trial using acne grade and lesion counts to monitor progress. Since it is likely that

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azelaic acid exerts its action, at least in part, through an antimicrobial effect (Nazzaro-Porro *et al.*, 1983), density of cutaneous microflora was also assessed. In addition, azelaic acid may affect sebaceous gland function (Breathnach, Nazzaro-Porro & Passi, 1984) so measurements of sebum excretion rate (SER) were made on a separate group of subjects.

## METHODS

### *Group 1*

Forty-five male acne patients were investigated between November and May. None had used acne treatment for 6 weeks before entering the study. Clinical and microbiological assessments were made prior to treatment and after 1, 2, 4 and 6 months of treatment. Twenty-three patients (Group 1A) were given placebo tablets and 20% azelaic acid cream.* Twenty-two patients (Group 1B) were given oral tetracycline (250 mg q.d.s.; tablets being taken with water 30 min before food) and placebo cream. Topical therapy in Groups 1 and 2 (see below) was applied liberally twice a day to face, back and chest. There was no double (tablets and cream) placebo group.

Clinical assessment was carried out by grading the acne and counting the lesions (Burke & Cunliffe, 1984). The density of micro-organisms on the skin was determined by the method of Cove, Cunliffe & Holland (1980). Side effects were assessed at each visit.

### *Group 2*

A group of 11 male subjects with physiological acne were treated with topical 20% azelaic acid over the same period as Group 1. No subject had used acne treatment for 6 weeks before starting the trial. Sebum excretion rate was estimated by the gravimetric technique (Strauss & Pochi, 1961; Cunliffe & Shuster, 1969) before treatment and after 1, 2, 4 and 6 months of treatment.

TABLE 1. Group 1, patient details and pre-treatment acne grades and lesion counts

| | A | B |
|---|---|---|
| | 20% azelaic acid (topical) | oral tetracycline (1 g/day) |
| No. of patients entering trial | 23 | 22 |
| Mean age (years) | 18 | 19 |
| No. of patients completing trial | 17 | 22 |
| Acne grade; median (range) | | |
| Facial | 2·0 (0·3–6·0) | 1·8 (0·2–4·0) |
| Total | 3·8 (1·5–9·8) | 2·6 (0·5–9·3) |
| No. of lesions; median (range) | | |
| Non-inflamed | 52 (26–130) | 41 (12–126) |
| Inflamed | 44 (13–127) | 43 (14–116) |

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* Details of the formulation of this cream are not currently available for publication for reasons of patent protection.

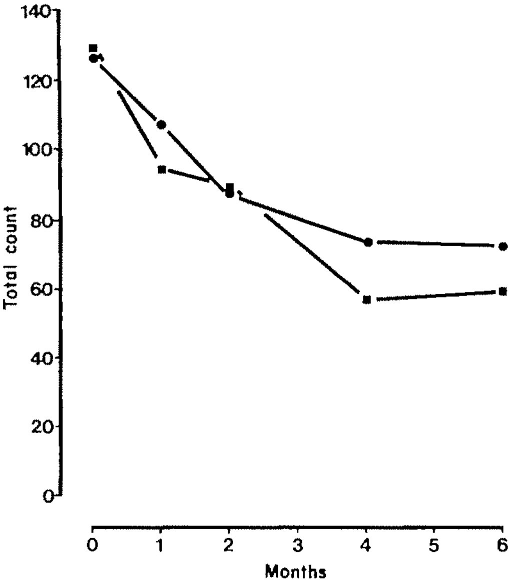

FIGURE 1. The effect of azelaic acid (●) and of tetracycline (■) on total lesion counts during 6 months' treatment. Points are medians.

### *Statistical analysis*

The Mann Whitney U test was used to evaluate clinical data (total lesion, inflamed lesion and non-inflamed lesion counts and acne grade) obtained from the different treatment groups. Intra-group comparisons were made using the Wilcoxon matched-pairs signed rank test. Analysis of laboratory data was carried out using Student's *t*-test.

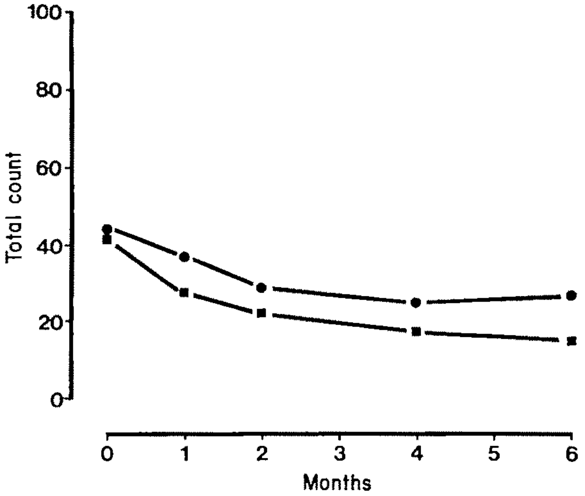

FIGURE 2. The effect of azelaic acid (●) and of tetracycline (■) on numbers of inflamed lesions (papules, pustules, deep pustules and nodules) during 6 months' treatment. Points are medians.

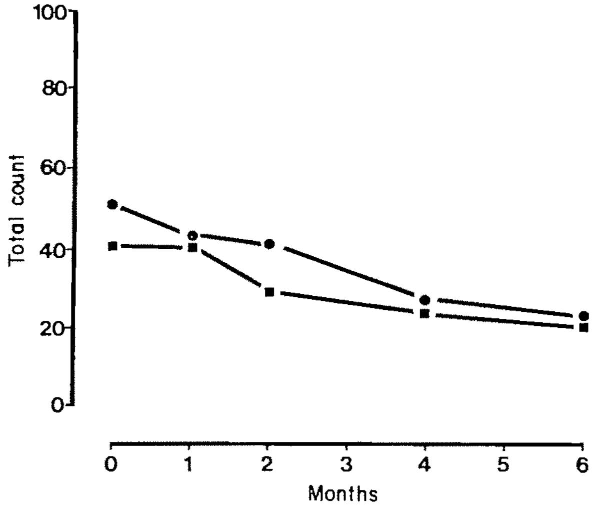

FIGURE 3. The effect of azelaic acid (●) and of tetracycline (■) on numbers of non-inflamed lesions (blackheads and whiteheads) during 6 months' treatment. Points are medians.

## RESULTS

### *Group 1*

The treatment code was broken after analysis of the results. Six patients who had been given azelaic acid (Group 1A) left the study prematurely (Table 1). One patient left for no given reason and five patients left because of poor response/compliance to the treatment. Of these patients four were subsequently given oral tetracycline (1 g/day) and 5% benzoyl peroxide cream and responded well; one was given oral 13-*cis* retinoic acid and also responded satisfactorily. No patients left the tetracycline treated group (Group 1B).

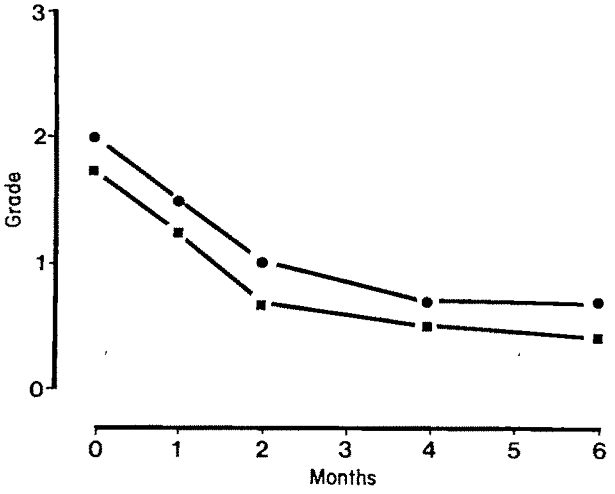

FIGURE 4. The effect of azelaic acid (●) and of tetracycline (■) on facial acne grades during 6 months' treatment. Points are medians.

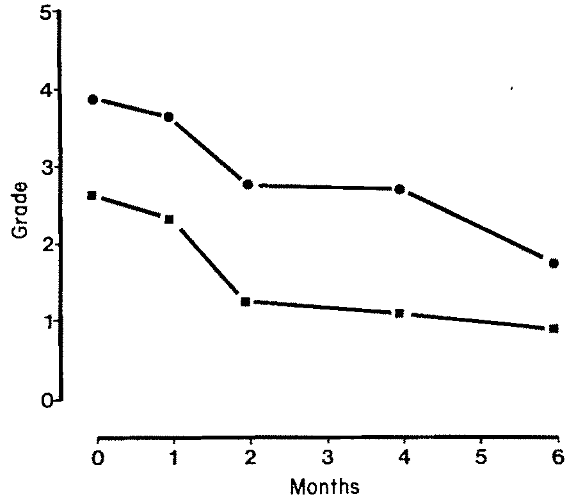

FIGURE 5. The effect of azelaic acid (●) and of tetracycline (■) on total acne grades during 6 months' treatment. Points are medians.

Azelaic acid and oral tetracycline each produced a significant decrease in total lesion count after a 4-month period (Fig. 1) (azelaic acid $P < 0{\cdot}01$, tetracycline $P < 0{\cdot}001$) and this was sustained to the sixth month. There were significant reductions in inflamed lesions (Fig. 2) after 2 months in both treatment groups and in non-inflamed lesions (Fig. 3) after 2 months in the tetracycline treated group and after 4 months in the azelaic acid treated group. After 6 months the median facial acne grade (Fig. 4) was decreased by 66% in the azelaic acid treated group, and by 77% in the tetracycline treated group. Inter-group analysis of total acne grade for face, back and chest (Fig. 5) showed a significantly lower median grade in the tetracycline group compared with the azelaic acid group after 4 and 6 months ($P < 0{\cdot}05$). Inter-group analyses of all clinical data are summarized in Table 2.

TABLE 2. Group 1; intergroup comparison of patients treated with azelaic acid and with tetracycline

| | Time of assessment (months) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 6 |
| Comedones | ns | ns | ns | ns | ns |
| Papules and pustules | ns | ns | ns | ns | ns |
| Nodules and deep pustules | ns | ns | ns | $0{\cdot}1 > P > 0{\cdot}05$ | $0{\cdot}1 > P > 0{\cdot}05$ |
| Macules | ns | ns | ns | $0{\cdot}1 > P > 0{\cdot}05$ | $P > 0{\cdot}1$ |
| Total lesions | ns | ns | ns | $0{\cdot}1 > P > 0{\cdot}05$ | $P > 0{\cdot}1$ |
| Facial acne grade | ns | ns | $P < 0{\cdot}05$ | $P > 0{\cdot}1$ | $0{\cdot}1 > P > 0{\cdot}05$ |
| Total acne grade | ns | ns | $0{\cdot}1 > P > 0{\cdot}05$ | $P < 0{\cdot}05$ | $P < 0{\cdot}05$ |

ns = not significant.

Azelaic acid reduced the density of the skin microflora, the main effect being a 224-fold reduction in the numbers of micrococcaceae. After one month, azelaic acid had produced a significant reduction in the density of micrococcaceae ($P<0.001$). A further significant reduction was observed after the second month in comparison with the first ($P<0.025$) and thereafter there was no further decrease. Tetracycline had no significant effect on the density of micrococcaceae. Azelaic acid produced on 30-fold decreacse in the density of *Propionibacterium sp.* on the skin but no decrease was detected with tetracycline treatment.

Side effects were seen in both treatment groups. These were transient and did not cause the withdrawal of any patient from the study. Minor erythema and scaling was observed in some patients receiving azelaic acid. One patient in the tetracycline group reported abdominal pain.

### *Group 2*

Azelaic acid had little effect on the SER. The SER did not differ significantly from the baseline value at the 1, 2 and 6-month visits. The mean SER at the control visit was $0.89 \pm 0.09$ μg/cm$^{-2}$/min$^{-1}$ (95% confidence limit) and at 6 months was $0.92 \pm 0.62$ μg/cm$^{-2}$/min$^{-1}$. However, there was a significant but very small decrease in SER at the 4-month visit, mean SER $0.84 \pm 0.57$ μg/cm$^{-2}$/min$^{-1}$ ($P<0.025$).

## DISCUSSION

Azelaic acid (1,7-heptane dicarboxylic acid, $HO_2.C(CH_2)_7 \cdot CO_2H$) has been reported in an open study to be of value in the treatment of moderate and severe acne (Nazzaro-Porro *et al.*, 1983). The present study showed that azelaic acid was beneficial in patients with clinical acne. However, it did not appear to be as effective as was reported by Nazzaro-Porro *et al.* (1983), and was found to be clinically marginally less effective than oral tetracycline. It had few side effects.

The laboratory studies showed that azelaic acid had an antimicrobial effect and that this was mainly on micrococcaceae. In comparison, tetracycline had no significant effect on the skin microflora. The mechanism by which tetracycline is effective in the treatment of acne is still open to discussion. Direct antibacterial effects have been demonstrated using 1000 mg daily, which reduced the cutaneous microflora (Marples & Kligman, 1971). At a lower dose of 250 mg daily, indirect effects on the microflora have been shown by Cunliffe *et al.* (1973) who argued that the reduction in skin surface free fatty acids was due to the lower production of lipase by cutaneous bacteria. However, tetracycline has a wide range of activities and lowers inflammatory responses by affecting non-immunological and immunological systems (Eady, Holland & Cunliffe, 1982b). Azelaic acid had little effect on SER and this finding is in agreement with that of Marsden & Shuster (1983). During these studies it was not possible to include a placebo group and it is hoped in future studies that this control can be included.

We conclude that azelaic acid has some potential in the treatment of mild or physiological acne. Further work on delivery systems, and its mechanism of action, could lead to the successful use of azelaic acid as a topical treatment for acne vulgaris.

## ACKNOWLEDGMENT

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# Hydroquinone concentrations in skin lightening creams

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## SUMMARY

Forty-one over-the-counter skin lightening creams were analysed for hydroquinone content, and the accuracy of tables of contents supplied with these products was assessed. Eight of the 41 were found to contain more than 2% hydroquinone, the maximum concentration permitted by the U.K. Cosmetic Products Regulations. Eighteen of these preparations failed to comply with labelling requirements. A list is given of those products containing less than 2% hydroquinone which are adequately labelled.

Skin bleaching or lightening creams are widely advertised and extensive markets for these products exist in South Africa, Nigeria and in the black communities of Europe and the United States. The active constituent of most skin lighteners is hydroquinone (*p*-dihydroxybenzene) which inhibits tyrosinase and prevents the conversion of tyrosine to dihydroxyphenylalanine, a precursor of melanin. Engasser and Maibach (1981) consider that hydroquinone is the only relatively safe and effective agent for lightening skin pigment.

During 1984 questions were raised concerning the safety of skin lightening preparations and how effectively regulations controlling the content and labelling of these products were being enforced (Ridley, Adams & Leigh, 1984; Boyle & Kennedy, 1984). The U.K. Cosmetic Products Regulations (1978) require that hydroquinone should be present in a concentration of 2% or less in skin lightening creams, and that a warning to avoid contact with the eyes must be carried on the product which should be labelled as containing hydroquinone.

As a result of these reports, a comprehensive study of the composition and labelling of skin lightening products available to the black community was undertaken by the Department of Trade and Industry and also by Lambeth Borough Council.

## METHODS

Forty-one skin lightening creams, freely available over the counter, were tested by the

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Department of Trade and Industry and Lambeth Borough Council to determine the concentration of hydroquinone present in each of the creams. The Laboratory of the Government Chemist analysed the samples for the Department of Trade and Industry using a previously unpublished method (Rix, in press). A firm of analytical and consulting chemists undertook the analysis for Lambeth Borough Council using the method outlined below.

The concentration of hydroquinone present in the various creams was determined using high pressure liquid chromatography. A 250 × 4 nm Li Chrosorb Si 60 (5 μm) column fitted with a Whatman HC Pellosil guard column and an LDC Spectromonitor III Model 120 4A variable wavelength detector was used. The eluent comprised chloroform 400 volumes, cyclohexane 50 volumes, absolute alcohol 50 volumes and 0·880 ammonia 1 volume. Eluent flow rate was 1·50 $cm^3$/min. The detector wavelength was 290 nm and detector range was 0·2 Absorbance units full scale. Chart speed was 4mm/min and chart range was 10 nV. The first 3 cm of cream extruded from the tube was discarded. 30 mg L-ascorbic acid and 0·3 g of the cream were placed in a dry 25 $cm^3$ volumetric flask, previously flushed out with nitrogen. 15 $cm^3$ of industrial methylated spirit (IMS) was added to dissolve the sample and 4 drops 0·880 ammonia were added and made up to the mark with IMS. The sample was filtered through a 0·8 μm membrane filter. The clear filtrate was collected and a 10 μl aliquot was injected onto the HPLC column.

Standard solutions were prepared by weighing 25 mg of L-ascorbic acid and 5, 8 and 10 mg of hydroquinone into 25 $cm^3$ volumetric flasks, previously flushed out with nitrogen. 15 $cm^3$ of IMS was added and shaken to dissolve. 4 drops of 0·880 ammonia were added and made up to the mark with IMS. 10 μl aliquots were injected onto the column. Replicate injections were made and, using the peak heights obtained for the standard solutions, a graph of concentration versus peak height was plotted. The concentration (C) of hydroquinone was read off using the peak height obtained for the sample solution. The percentage concentration of hydroquinone was calculated as below where C is in p.p.m. and *w* is the weight of sample taken.

$$\%\ \text{Hydroquinone} = C \times \frac{25}{w} \times \frac{1}{10}$$

## RESULTS

Eight skin lightening creams were found to contain more than 2% hydroquinone (Table 1) and

TABLE 1. Products containing more than 2% hydroquinone

| | LBC | DTI |
|---|---|---|
| Ambi extra complexion cream (double strength) | 5·8% | 5·0% |
| Ambi extra for men complexion cream | 7·8% | 7·0% |
| Ambi skin cream (for oily skin) | 6·0% | 6·0% |
| Ambi special complexion cream | 5·0% | 5·0% |
| Clear skin toning cream | 4·3% | 4·0% |
| Envi skin toner | 5·0% | 5·0% |
| Paulina beauty cream | 5·8% | 6·0% |
| Venus de Milo complexion cream (Nigerian) | 5·7% | 5·0% |

LBC = Concentration determined by Lambeth Borough Council.

DTI = Concentration determined by Department of Trade & Industry.

TABLE 2. Products failing to comply with labelling requirements

| |
|---|
| Ambi extra complexion cream (double strength) |
| Ambi extra for men complexion cream |
| Artra skin tone cream (for dry/normal/oily skin) |
| Black and white bleaching cream |
| Clear tone |
| Dorot medicated skin cream |
| Envi skin toner |
| Fred Palmer's skin whitener |
| Nadinola skin cream |
| Nadinola deluxe |
| Nadinola ultra |
| Palmer's skin success cream |
| Paulina beauty cream |
| Skin tona cream |
| Ultra bleach and tone cream |
| Venus de Milo cream (Nigerian) |

TABLE 3. Products complying with regulations on hydroquinone concentration and labelling

| |
|---|
| Ambi extra skin toning cream |
| Ambi special skin toning cream |
| Ambi skin cream for dry skin |
| Ambi skin tone cream for normal skin |
| Esoterica (facial) medicated fade cream |
| Esoterica (with sunscreen) medicated fade cream |
| Esoterica (regular) medicated fade cream |
| Fade out effective treatment cream |
| Symba skin-lite'n smooth cream |
| Ultra glow skin tone cream |
| Ultra glow skin tone cream and sunscreen |
| Venus de Milo cream (UK) |

18 of the products failed to comply with labelling requirements (Table 2). There was close correlation between the results of the analyses undertaken in the two laboratories and no result differed by more than 0·8%. Products complying with the labelling requirements and containing 2% hydroquinone or less are shown in Table 3.

## DISCUSSION

Hydroquinone is considered relatively safe if used in low concentrations in treating melasma or post-inflammatory hyperpigmentation. The incidence of side effects (allergic contact and irritant dermatitis) is greatly increased, however, at higher concentrations (Engasser & Maibach, 1981). The combination of hydroquinone with tretinoin, salicylic acid or topical steroids—the latter to reduce the incidence of dermatitis caused by the higher concentrations of

TABLE 4. Products containing mono methyl ether of hydroquinone

| | |
|---|---|
| Drula bleaching wax | 1·5% |
| Drula bleaching wax—extra concentrated | 2·0% |
| Venus de Milo cream (UK) | 1·0% |

hydroquinone—has also been suggested (Kligman & Willis, 1975; Engasser & Maibach, 1981). Hydroquinone 2% and tretinoin 0·1% in an alcoholic lotion was found to be effective in the treatment of melasma by Pathak, Sanchez & Fitzpatrick (1980). Use of sunscreens during and after treatment is also important to prevent repigmentation.

Other chemicals are capable of depigmenting skin. These include monobenzyl ether of hydroquinone which is both potent and unpredictable. Its use in cosmetics is prohibited (Cosmetic Products Regulations, 1978). The monomethyl ether of hydroquinone is also used as a skin lightening agent especially in Europe and Africa but unsightly depigmentation associated with its use has been reported (Colomb, 1982: Boyle & Kennedy, 1985). This agent was detected in three skin lightening creams (Table 4) although it is no longer present in one (Venus de Milo cream).

Rather surprisingly, the monomethyl ether of hydroquinone, despite being more potent than hydroquinone (Brun, 1959) does not appear in the Cosmetic Products Regulations (1978) as a prohibited or controlled substance, but the EEC Cosmetic Working Party has now recommended that the monomethyl ether of hydroquinone should be prohibited from use in cosmetics and skin lightening preparations.

Officers of the Trading Standards Department have been advised of the products failing to comply with the regulations and traders have been asked to withdraw the offending products voluntarily or face prosecution.

## ACKNOWLEDGMENT

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***Case Report***

# Two cases of adult T-cell leukaemia

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## SUMMARY

A 70-year-old male and a 46-year-old female, both living in Japan, were seen because of multiple erythematous papules and plaques on the trunk and extremities. The peripheral white blood cell counts were increased to 30 000–80 000/mm$^3$. The majority of white cells had characteristic lobulated and deeply indented nuclei. The patients and most of their family members had anti-ATLV antibody. Using an OKT series of monoclonal antibodies the tumour cells were found to be T-cells. Trisomy 21 was detected in the first case and ATL virus proviral DNA in the second.

The concept of adult T-cell leukaemia (ATL) was first suggested by Takatsuki *et al.* (1976). The disease is characterized by poor prognosis, the clustering of patients' birthplaces in the Kyushu district of Japan, leukaemic cells with deeply indented nuclei, and frequent skin involvement (Uchiyama *et al.*, 1977). In addition, clusters of the disease in the Caribbean basin have been recently reported (Catovsky *et al.*, 1982; Blattner *et al.*, 1983). Few clinical articles on ATL have appeared in dermatological journals.

## CASE I

A 70-year-old man living in Oita Prefecture, Kyushu, developed asymptomatic diffuse erythema on the trunk and lower limbs and erythematous papules on the upper limbs over a 2-week period in October, 1981, and was admitted to the dermatological clinic of the Medical College of Oita in November, 1981. He had a 10-year history of hypertension. His family history was unremarkable.

Physical examination on admission showed slight hepatosplenomegaly and lymph node enlargement in both inguinal regions. Numerous erythematous papules coalesced into irregularly-shaped plaques on both upper limbs (Fig. 1). Most of the trunk and both lower limbs showed ill-defined erythema. A biopsy specimen taken from an erythematous papule on the

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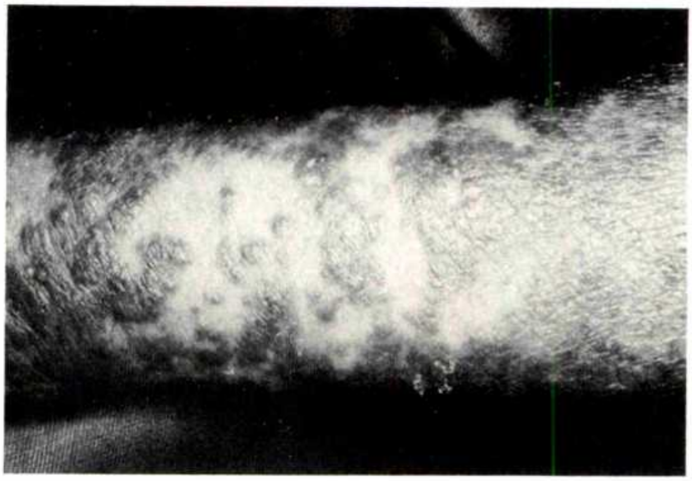

FIGURE 1. Dark red, coalescent papules on the upper limb (Case 1).

back of the right hand showed a dense, band-like infiltrate of mononuclear cells, (Fig 2a), that varied in nuclear configuration (Fig. 2b). Infiltration of these cells into the epidermis was seldom observed in this specimen, but the plaque on the abdominal wall showed marked epidermotropism of atypical mononuclear cells. The normal architecture of the lymph node from the

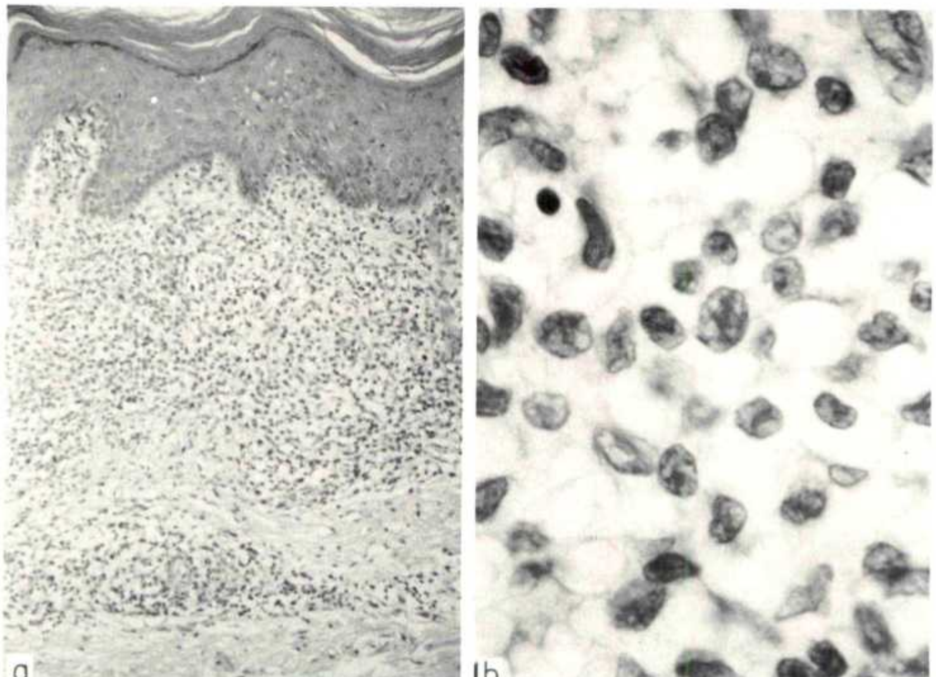

FIGURE 2. (a) Band-like infiltration in the upper dermis and (b) irregularly-shaped nuclei of varying stainability (Case 1). (H & E, original × 40 and × 400).

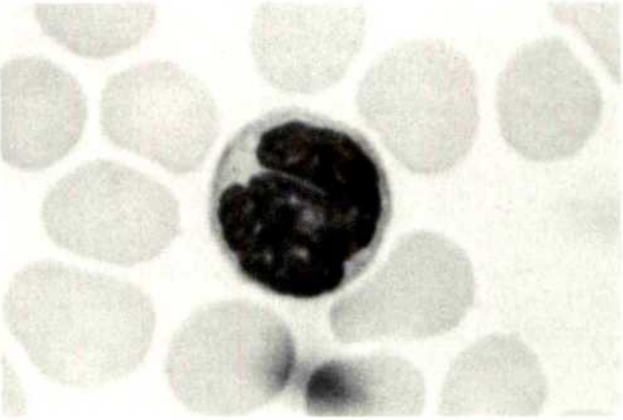

FIGURE 3. Atypical peripheral lymphocytes with lobulated nuclei (Case 1). (Giemsa, original × 1000).

inguinal region was completely effaced as a result of massive tumour cell infiltration. The nuclei of the tumour cells were large, irregularly shaped and pleomorphic, with prominent nucleoli.

The laboratory findings on admission were: haemoglobin 17·8 g/dl, WBC count 18 600/mm$^3$ with 35% neutrophils, 27% lymphocytes, 4% monocytes, and 33% atypical mononuclear cells. The platelet count and serum IgG, IgA and IgM levels were within normal limits. The patient was PPD positive. The bone marrow aspirate showed many pleomorphic blast cells (54·8%). E-rosette and EAC-rosette-forming cells accounted for 83·5% and 1·7% of peripheral

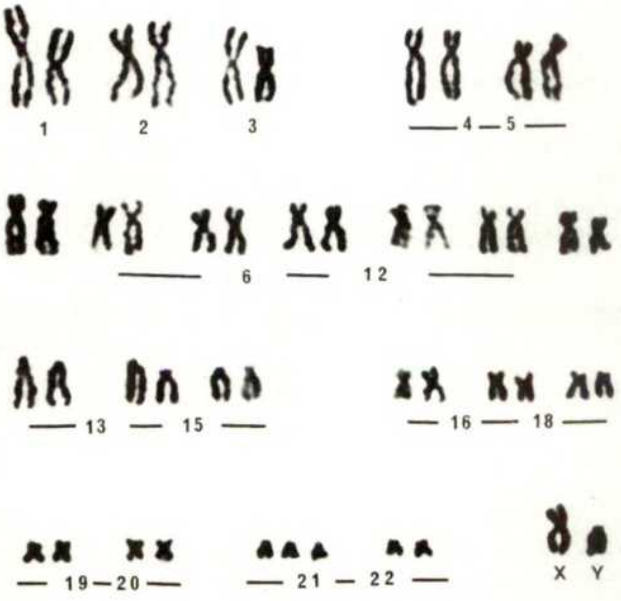

FIGURE 4. Chromosomal mode of tumour cells in the peripheral blood. Note trisomy 21.

lymphocytes, respectively. The nuclei of the atypical peripheral lymphocytes were lobulated and deeply indented, with abundant cytoplasm (Fig. 3).

Laboratory findings specifically related to ATLV were as follows: the patient, his wife and two of his three children had anti-ATL antibody. The lymphocyte phenotype of the atypical mononuclear cells was: SIg 2·5%, OKT1 10·2%, OKT3 92·7%, OKT4 96·8%, OKT8 4·5% and Tac 12·7%. Chromosome analysis of cultured lymphocytes revealed a hyperdiploid pattern with trisomy at chromosome 21 (Fig. 4).

A week after admission, the WBC count increased to 86 000/mm$^3$ with 77% atypical cells. Six weeks of combination therapy 40 mg prednisolone daily, 1 mg vincristine weekly and 300 mg cyclophosphamide weekly, led to a decrease in WBC count to 30 000–40 000/mm$^3$ with 30–40% atypical cells. The erythema on the trunk and both lower limbs faded and the erythematous papules flattened. The drug dosage was therefore reduced in the 25th week of treatment, but this resulted in an increase of total WBC to 74 000/mm$^3$ with 85% atypical cells. Re-institution of therapy at increased dosages failed to give any beneficial effects and the patient died of aspiration pneumonia in the 41st week of hospitalization. Autopsy revealed leukaemic cell infiltration in the bone marrow, spleen, lung, kidney, oesophagus, stomach, colon, urinary bladder and lymph nodes, a leiomysarcoma of the right kidney, and cytomegalic inclusion bodies in the oesophagus, lung and urinary bladder.

## CASE 2

A 46-year-old woman, from Oita, presented with a widespread non-pruritic skin eruption first noticed on the right lower leg in December, 1983. Two months later, erythematous papules and plaques involved almost the whole body surface.

She was referred to the dermatological clinic of the Medical College of Oita in February, 1984. Physical examination revealed numerous, well-defined, fresh red papules and plaques on the whole body except for the head, palms and soles (Fig. 5). They were moderately scaly and some were annular. She was clinically jaundiced. The liver and spleen were both enlarged 4 cm below the costal margin. There was lymph node enlargement in both inguinal regions. A biopsy specimen of an erythematous plaque from the abdominal wall showed the upper dermis densely infiltrated by numerous mononuclear cells. Most nuclei were hyperchromatic and convoluted. The epidermis was invaded by scattered atypical mononuclear cells with a halo-like clear space (Fig. 6). In the inguinal lymph node a massive infiltration of atypical lymphoid cells was observed as in Case 1. The laboratory findings were: WBC count 33 100/mm$^3$ 40% atypical

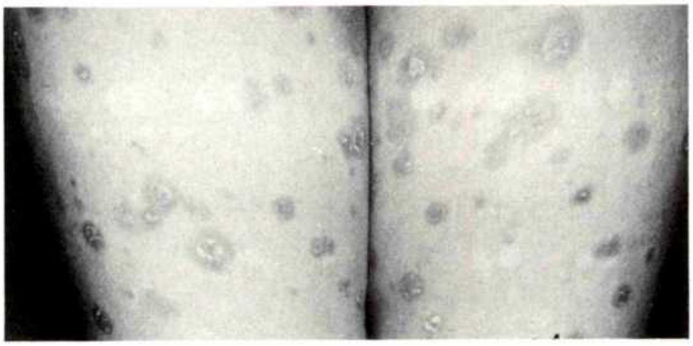

FIGURE 5. Well-defined, fresh red, round plaques of various sizes are scattered on the thigh (Case 2).

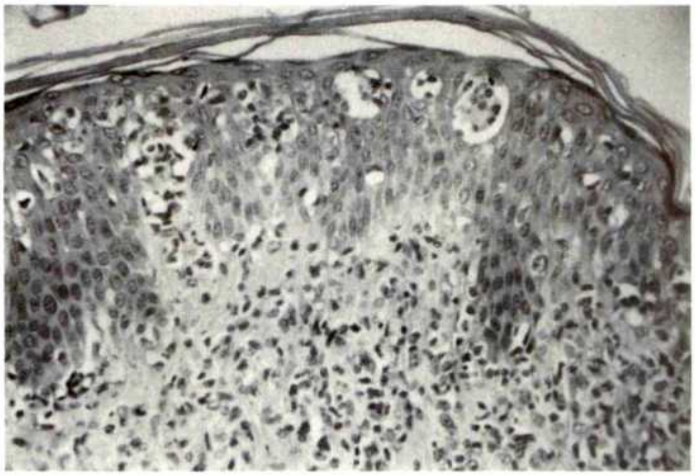

FIGURE 6. Groups of irregularly-shaped lymphoid cells invading the epidermis resemble Pautrier micro-abscesses (Case 2). (H & E, original × 200).

monocytoid cells, total bilirubin 6·3 mg/dl (0·1–1·1), SGOT 3306 U (11–36), SGPT 1520 U (2–34), alkaline phosphatase 1718 IU/l (82–240) and LDH 2587 IU/l (212–410). Serum calcium and immunoglobulins were within normal limits.

Laboratory findings related to ATL were as follows: the patient, her mother, brother and husband were ATL antibody positive, while her sister and two daughters were negative. The lymphocyte surface phenotype was: OKT3 76·3%, OKT4 85·1%, OKT8 6·1%, OKT11 95·2%, polyIg 1·7%, $\kappa$ 0·3% and $\lambda$ 0·2%. Seventy two percent of peripheral white blood cells were T cells and 4% B cells, when analysed by rosette formation.

A panel of monoclonal reagents was used to identify T and B cells in the lymph node. As shown in Figure 7, most tumour cells were positive for anti-Leu-1 and -4 and only a small

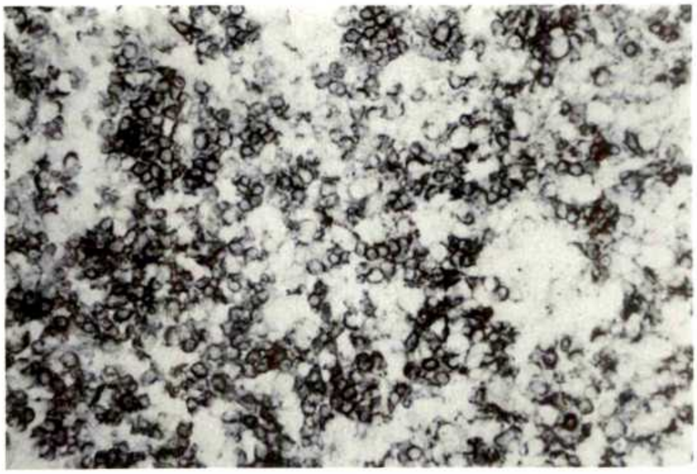

FIGURE 7. A specimen of the lymph node (Case 2) showing the predominance of anti-Leu-1 positive cells (immunoperoxidase method). (Original × 100).

proportion positive for anti-Leu-10. Thus, most of the tumour cells infiltrating the lymph nodes were T cells as in the peripheral blood. Leukaemic cells were found to contain ATL virus proviral DNA integrated in cellular DNA, by the modified Southern blotting method of Yoshida *et al.* (1984).

In March, 1984, combination therapy similar to the regimen in Case 1 was started. After one and a half months, the WBC count was decreased to 5900/mm$^3$ with 10% atypical lymphoid cells, the liver function tests returned to near- normal levels, and the skin eruption markedly improved. Treatment had to be stopped for 2 months because of bleeding from a gastric ulcer and disseminated intravascular coagulation. Although the proportion of atypical cells in the 10 000 WBC count was reduced to 20%, numerous scaly erythematous plaques were resistant to further chemotherapy and PUVA treatment. The patient died of intestinal bleeding in January, 1985. Autopsy revealed a leukaemic cell infiltration in the bone marrow, spleen and hilar, tracheal and mesenteric lymph nodes. She also had pulmonary aspergillosis, cytomegalic infection in the lung, adrenal gland, stomach, jejunum, ileum and colon, and ulcers in the ileocaecal area and stomach.

## DISCUSSION

One of the characteristic features of ATL is the frequent manisfestation of skin eruptions which are recognizable as erythroderma and erythematous papules, nodules and plaques. These were noted in half of the previously reported cases (Uchiyama *et al.*, 1977; Takatsuki *et al.*, 1979). Although other cutaneous T-cell lymphomas such as Sézary syndrome and mycosis fungoides may present similar skin lesions or histological features, most cases of ATL have a more acute and unfavourable course. It is therefore of vital importance to differentiate ATL from other cutaneous T-cell leukaemias/lymphomas and commence treatment rapidly.

Patients previously reported with ATL were mostly natives in the Kyushu district, especially in seaside villages or small islands (Takatsuki *et al.*, 1979). This clustering suggested a viral involvement in the pathogenesis of ATL. Recently ATL virus (ATLV), a member of human T-cell leukaemia virus group (Popovic *et al.*, 1982), was detected in the cultured lymphocytes from ATL patients (Hinuma *et al.*, 1981; Poiesz *et al.*, 1980). The additional detection of ATLV proviral DNA in peripheral blood mononuclear cells strongly supports the viral origin of the disease (Yoshida *et al.*, 1984). All reported cases of ATL, including our cases, and most of their family members had anti-ATLV antibody. The presence of ATLV-associated antigen and antibodies in other T-cell leukaemias/lymphomas has been reported by Gallo *et al.* (1983). Only two of 251 patients with cutaneous T-cell lymphoma in the USA or Europe were positive for ATLV antibodies, whereas Amagasaki *et al.* (1984) stated that more than 80% of patients with T-cell leukaemia/lymphoma other than ATL were positive for both the antigen and antibody. The OKT3+ and OKT4+ phenotype of our two cases suggests that the tumour cells arose from the helper/inducer subset of T cells. In addition, in Case 2, the tumour cells in the lymph node were also T cells with anti-Leu-1 and -4 positivity.

To date, only a few chromosomal studies have been performed on T-cell neoplasms. Ueshima *et al.* (1981) reported that 14 of 15 patients with ATL had chromosomal abnormalities, trisomy 7 being most frequent. The significance of these chromosomal abnormalities is not yet clear.

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### Wallace's Line

MADAM, Drs Harper and Copeman presented a poster entitled 'Wallace's Line, what is it?' at the 1983 Annual Meeting of the British Association of Dermatologists. They noted, as had the late Dr H.J.Wallace of St. Thomas' Hospital, that certain dermatoses, such as lichen planus and pompholyx, are delimited sharply at the medial and lateral borders of the hands and feet. In their poster, they considered the possible underlying reasons for the existence of Wallace's Line. Sweat glands, dermatoglyphics, hair and, in the darker races, pigmentation, all alter sharply at Wallace's Line. On the basis of three observations a hypothesis is proposed.

(i) Sappey (1874) traced the pathways of the cutaneous lymphatic system. Figure 1 is a plate from his book. It can be seen clearly that the lymphatic pattern sharply alters at Wallace's Line. The transitional zone runs along the medial and lateral borders of the feet and, like Wallace's Line, forms a spur reaching proximally, two or three finger-breadths, from the insertion of the Achilles tendon: the lymphatics being more plentiful on the sole than the dorsum, an observation confirmed by Yoffey and Courtice (1970).

(ii) Figure 2 shows a severely ecchymotic foot in which swelling and discolouration are limited to the dorsal surface. Distal to Wallace's Line, where the lymphatics are plentiful, drainage has been rapid and efficient.

(iii) Statis ulceration never occurs distal to Wallace's Line.

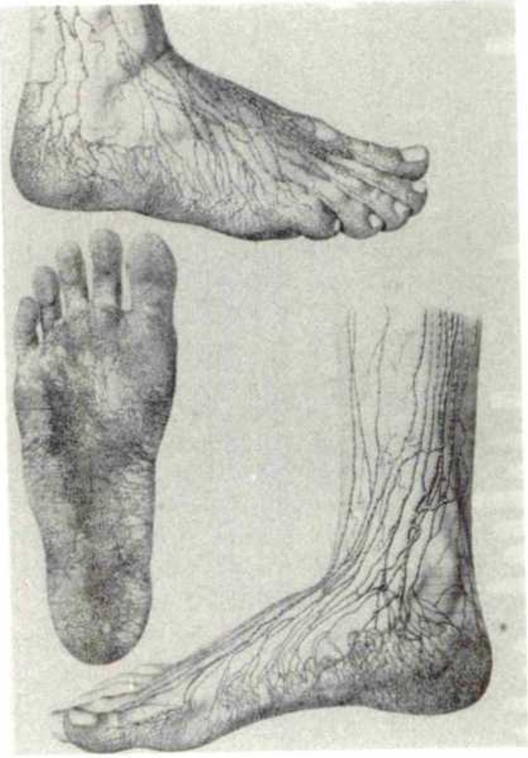

FIGURE 1. The lymphatic drainage of the skin of the foot (reproduced from Sappey, 1874).

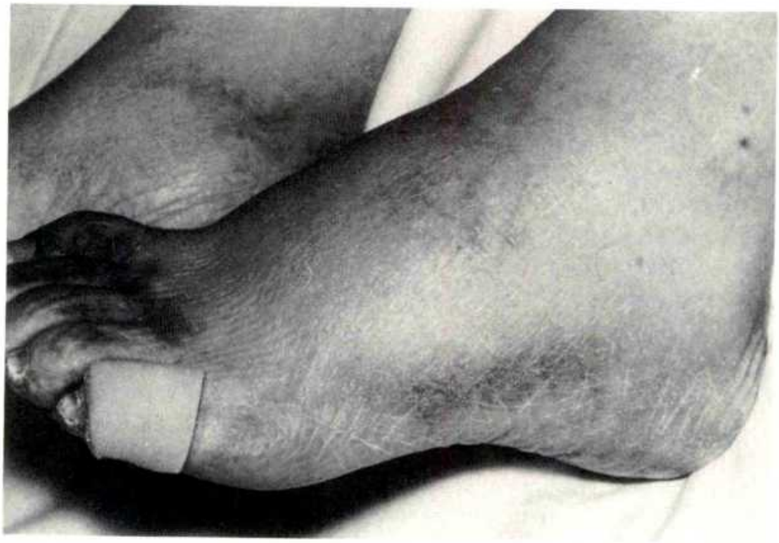

FIGURE 2. A severely bruised foot.

We propose that, in disease, Wallace's Line owes more to the pattern of the lymphatic drainage of the skin than to any other anatomical factor, and suggest that the poorer drainage of the dorsal surfaces delays clearance of unwanted material such as interstitial fluid and antigens.

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## Association between lichen sclerosus et atrophicus and primary biliary cirrhosis

MADAM, We wish to comment on the letter from Dr Lavery and colleagues (*British Journal of Dermatology*, 1985, **112**, 729) reporting an association between lichen sclerosus et atrophicus (LSA) and primary biliary cirrhosis (PBC). This preliminary report is based on their detection of PBC in five out of 100 patients with LSA, as evidenced by finding smooth muscle antibodies in one patient with LSA and generalized pruritus, 'serological evidence of PBC' in three other patients with LSA, and LSA in one patient 'with PBC'; no corroborative evidence of abnormalities of either liver function or liver histology is provided to support the diagnosis of PBC in any of the five patients.

Dr Lavery and colleagues cite smooth muscle antibodies as evidence for a provisional diagnosis of PBC. However, the presence of antimitochondrial antibody in the serum is the serological marker for PBC (Walker *et al.*, 1965) whereas smooth muscle antibodies are associated with chronic active hepatitis and are usually absent from the serum of patients with PBC (Whittingham *et al.*, 1966). In order to uphold an association of LSA with PBC (or chronic active hepatitis), we believe that the authors should have provided evidence of deranged liver function and liver histology supportive of the diagnosis in these patients. As both smooth muscle antibodies and antimitochondrial antibodies occur in the general population (Hawkins *et al.*, 1979), serological markers alone cannot be relied upon as proof of liver disease; furthermore, smooth muscle antibodies have been shown to disappear over a 6-year period from the serum of more than three quarters of those individuals in the general population in whom they had previously been detected (Hawkins *et al.*, 1979).

In our own study of 250 female patients with histologically confirmed LSA (Meyrick Thomas *et al.*, 1985), 19 patients demonstrated smooth muscle antibodies and four antimitochondrial antibodies (at a titre of 1 : 20 or greater). None of the 250 patients studied had developed autoimmune liver disease, and only two had experienced any other significant liver disease (one having alcoholic hepatitis and the other having suffered halothane-induced hepatitis following a general anaesthetic).

We do not believe that there is sufficient evidence at present to support the contention that there is an association between LSA and PBC.

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### REFERENCES

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## Reply

MADAM, With reference to the letter by Meyrick Thomas *et al.*, unfortunately we made an error in referring to smooth muscle antibodies, it should have been antimitochondrial antibodies. Four of the five patients refused liver biopsy and had no abnormality in routine liver function tests. The remaining patient had histological evidence of primary biliary cirrhosis. We reported these patients as showing a possible association between the conditions of primary biliary cirrhosis and vulval dystrophy, but appreciate that the evidence to date has not confirmed this relationship. However, we felt that the association may be of importance, as primary biliary cirrhosis is considered an autoimmune disease and vulval dystrophy has been shown to be linked with other autoimmune conditions, e.g. achlorhydria, pernicious anaemia and thyroid disease (Lavery, 1980).

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### REFERENCES

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## Comments on 'Conception during "Diane" Therapy—a successful outcome'

MADAM, Drs Statham, Cunliffe and Clayton reported (*British Journal of Dermatology*, 1985, **113**, 374) the delivery of a healthy full-term male infant, inadvertently conceived while the mother was taking Diane. In the opinion of the authors, the period of the foetus's exposure to cyproterone acetate ended before the differentiation of the external genitalia began, and they admitted that their case really threw no light on the possible existence of a risk of feminization when a male foetus is exposed to Diane at the critical period. The information that *was* relevant, however, and which was obtained from us, has not been given correctly in the letter. Moreover, it has been superseded by more recent information received from our parent company.

The current position is that we know of 13 male infants throughout the world who were exposed to Diane during the period of differentiation of the external genitalia, all of whom were diagnosed as normal healthy infants. We know of only one male born after exposure to the Hammerstein regimen, which because of a misunderstanding, was not withdrawn when pregnancy was diagnosed, and which was continued throughout the whole of the first half of pregnancy, i.e. throughout the entire period of organogenesis. I have been unable to ascertain whether the higher or lower dosage of the Hammerstein regimen was used, but this is really of no importance, since the foetus was exposed, throughout the period of genital differentiation and before it, to at least 25 times the daily dose of cyproterone acetate contained in Diane. In my view, the birth of a healthy male infant in that single case, which is unlikely ever to be repeated, provides vastly greater reassurance than the 13 healthy males who were exposed to Diane itself.

Obviously, this information should form part of any discussion with a patient who inadvertently conceives while taking Diane, and, while it is certainly reassuring, I fully agree with Drs Statham, Cunliffe and Clayton that it does not obviate the necessity of leaving the final decision about the continuation or termination of pregnancy to the patient, and to her husband, if she has one.

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P.BYE

## Association between bullous pemphigoid and psoriasis

MADAM, While we were interested in the article suggesting an association between bullous pemphigoid and psoriasis, written by Dr Grattan (*British Journal of Dermatology* 1985, **113**, 281), we wish to point out that the author did not refer to our paper describing eight patients with psoriasis who developed bullous pemphigoid after PUVA therapy (Brun & Baran, 1982).

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### REFERENCE

BRUN, P. & BARAN, R. (1982) Pemphigoïde bulleuse induite par la photochimiothérapie du psoriasis. *Annales de Dermatologie et Vénéréologie (Paris)*, **109**, 461.

## Cartilaginous naevus on the glabella

MADAM, We describe here a very rare case of a cartilaginous naevus, which developed on the glabella.

A 2-month-old boy was brought to our clinic with a pedunculated nodule on the glabella (Fig. 1). He had had a normal delivery and was otherwise normal. The nodule had been seen at birth, and its appearance and size had not altered. The patient had no family history of any cutaneous malformations. The nodule was the size of a bean, normal in colour and with the consistency of elastic tissue. It was excised totally under local anaesthesia. On histopathological examination a polyp-like structure covered with a normal epidermis was seen (Fig. 2). Young elastic cartilaginous tissue was found in the deep dermis.

Cartilaginous naevi develop as sessile or pedunculated polyps usually on the line between the pre-auricular region and the angle of the mouth and also, but less frequently, on the anterior part of the neck (Brownstein, Wanger & Helwig, 1971). The histopathological features are diagnostic, a central plate of

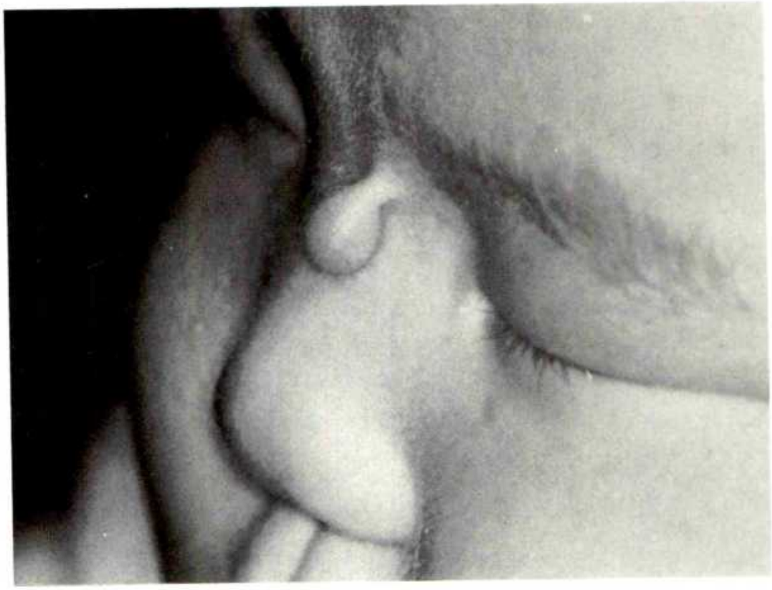

FIGURE 1. A pedunculated nodule on the glabella.

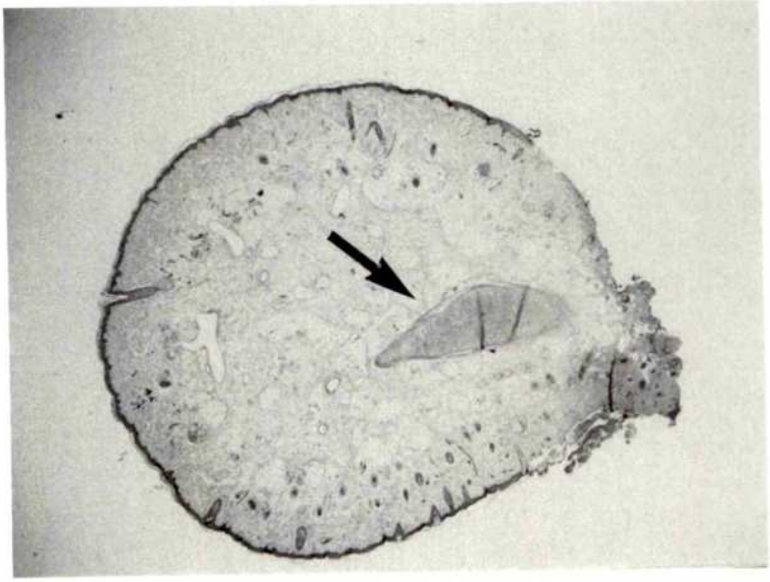

FIGURE 2. Histopathological section of the excised nodule (H & E stain). The arrow indicates cartilaginous tissue in the deep dermis.

cartilaginous tissue being present in the deep dermis. The histopathological features of the pedunculated nodule in our patient showed that it was a cartilaginous naevus. Cartilaginous naevis may develop, very rarely, on the glabella (Sayama & Tagami, 1982).

It has been suggested that a cartilaginous naevus on the glabella is a remnant of the branchial cartilage derived from the first branchial arch (Sayama & Tagami, 1982). However, the cartilaginous tissue of the nose is formed from the frontonasal prominence, and the muscles of facial expression are formed from the second branchial arch (Moore, 1977). Since the cartilaginous tissue of the nose is composed not of elastic, but of hyaline cartilages, the present cartilaginous naevus on the glabella may be derived from the second branchial arch rather than from the first branchial arch and the frontonasal prominence.

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ACKNOWLEDGMENT

The authors wish to thank Dr O.Tanaka, Department of Anatomy, Shimane Medical University, for his advice.

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## Short-contact dithranol therapy

MADAM, A recent report by Jones, Campbell and MacKie compared short-contact and overnight therapy with dithranol in patients with plaque psoriasis (*British Journal of Dermatology*, 1985, **113**, 331). Twelve patients in each treatment group completed the study. The authors concluded that there was no significant difference in the clearing achieved by the two regimes and that both were acceptable outpatient treatments except in those patients with skin of sun-reactive type I. Whilst agreeing with the main conclusions we feel that the statistical analysis merits further comment.

Physician assessment was by a scoring method using the psoriasis Area Severity Index (PASI) (Fredriksson & Pettersson, 1978) and patient assessment of staining and irritation scored on a four-point scale. In the data analysis the mean and SEM of the scores for each group are presented and statistical analysis was by paired and unpaired *t*-test. Student's *t*-test permits the testing of hypotheses with data which are drawn from a normal distribution of data values. A scoring index is not normally distributed. The central limit theorem, which is fundamental to statistics, states that such group means approximate to a normal distribution only if the groups are large (Armitage, 1971). Thus, to justify the use of a *t*-test in this case, it is clear that a sample size greater than 12 would be required. To quote *P* values is inappropriate and, indeed, why short-contact treatment caused an apparently significantly greater degree of irritation of perilesional skin than overnight treatment was unclear to the authors. We would suggest that their data are better presented as mean and standard deviation, and if statistical analysis is required then non-parametric methods be employed.

The conclusion that these treatment regimes were unsuitable for patients with skin type I does not appear to be justified by the data presented since only one patient of skin type I withdrew from the trial because of dithranol burning compared with three patients of skin type II.

May we look forward to more critical application of statistical tests by authors and referees so that good data collection is matched by good statistical analysis.

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REFERENCES

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FREDERIKSSON, T. & PETTERSSON, U. (1978) Severe psoriasis—oral therapy with a new retinoid. *Dermatologica*, 157, 245.

## Reply

MADAM, While we accept some of the criticisms offered above of our choice of statistical tests, the conclusions drawn from our study are unchanged.

In view of the fact that the PASI scoring system has possible scores ranging from 0–72 units, with increments of 0·1 units, we suggest that data obtained by this system does in fact conform to a normal distribution. We do concede, however, that paired and unpaired *t*-tests were not suitable for multiple analyses done on data obtained using the PASI scoring system, or for comparison of the patient irritation and staining assessment. We have re-analysed this data using Repeated Measures Analysis of Variance on the four PASI scores obtained for each patient, using the initial pre-treatment value as a covariate. This gave essentially similar results in that patients on both treatment regimes showed significant improvement in PASI scores over time ($P > 0\ 0001$).

When irritation and staining were compared using the Mann-Whitney test for non-parametric data, no significant differences between the two regimes were observed, although irritation of perilesional skin at 6 weeks, and staining of skin at 4 weeks, came very close to achieving significance ($0{\cdot}06 > P > 0{\cdot}05$).

As only one patient in our study had skin type I, and this patient showed burning with short contact dithranol, in contrast to only three out of 16 patients (19%) of skin type II who burned, we would hold to our conclusion that application of this method to patients with skin type I should be carried out with extreme caution.

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RONA M. MACKIE

## Pseudoporphyria due to naproxen

MADAM, We have recently seen a 52-year-old man who developed pseudoporphyria (the skin manifestations of porphyria cutanea tarda [PCT] with normal serum, urinary and faecal porphyrins) due to naproxen.

He had first developed spontaneous blisters on the dorsum of his hands and erosions on the scalp and forehead 2 years previously. The lesions had healed slowly with scarring. There was no relationship to sun exposure and no history of alcohol abuse. He had had a past episode of obstructive jaundice, and was taking naproxen 500 mg bd for mild arthritis in his hands. A porphyrin screen was repeatedly negative, but histology of a skin biopsy demonstrated extensive subepidermal bullae and no inflammatory infiltrate, consistent with PCT. Immunofluorescence showed a weak basement membrane band of IgG and IgM with perivascular C3 deposits suggestive of PCT. Liver function tests were normal and a liver biopsy showed non-specific inflammatory changes with no siderosis. In the presence of negative porphyrine estimations, the main alternative diagnosis was epidermolysis bullosa acquisita, but this has a characteristic immunofluorescence pattern (Yaiota *et al.*, 1981). A barium meal and follow through were normal.

Naproxen rarely causes a rash or uriticaria (Brogden *et al*, 1975) but recently its association with pseudoporphyria has been reported (Howard, Dowling & Varigos, 1985). The naproxen was stopped and the patient has remained in remission. Drug-induced pseudoporphyria is known to be associated with nalidixic acid, high dose frusemide and tetracylcine; naproxen is now also implicated.

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REFERENCES

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BROGDEN, R.N., PINDER, R.M. SAWYER, P.R. SPEIGHT, T.M. & AVERY, G.S. (1975) Naproxen: a review. *Drugs*, **9**, 326.

HOWARD, A.M., DOWLING, J. & VARIGOS, G. (1985) Pseudoporphyria due to naproxen. *Lancet*, **ii**, 819.

## Elevated serum aminoterminal procollagen type III peptide in methotrexate-induced hepatic fibrosis

MADAM, Long-term treatment with methotrexate may induce hepatic fibrosis, especially if the patient drinks alcohol. Until recently, hepatic fibrosis could only be detected by liver biopsy, which is associated with a small but definite morbidity and mortality. Recently, elevated serum aminoterminal procollagen type III peptide (PIIIP) concentrations have been found in patients with ongoing liver fibrosis (Hahn, 1984). RIIIP is a cleavage product of procollagen type III and is released into the extra-cellular fluid during the conversion of procollagen type III into collagen. Collagen type III is the predominant form of collagen deposited in the liver during hepatic fibrosis due to methotrexate. We have measured therefore, serum PIIIP concentrations in 17 patients receiving long-term treatment with methotrexate for severe psoriasis, who were attending for routine follow-up biopsies.

Thirteen patients had normal liver histology or only minor fatty change and four of these had elevated PIIIP concentrations (14, 15, 17 and 18 ng/ml respectively; normal range 3·12 ng/ml). One of the two patients with reactive changes in the liver had an elevated PIIIP concentration (14 ng/ml). Both of the patients with mild hepatic fibrosis had elevated PIIIP concentrations (16 and 17 ng/ml respectively). The seven patients with elevated PIIIP concentrations had received a larger total dose of methotrexate (mean 3770 mg, range 1640–6560 mg) than those with normal PIIIP concentrations (mean 1700 mg, range 220–8885 mg).

Our results suggest that measurement of the serum PIIIP may help predict those patients at risk of developing hepatic fibrosis while receiving long term methotrexate treatment for psoriasis. We are now undertaking a prospective study of the relationship between serum PIIIP concentrations and hepatic histology to verify these tentative conclusions.

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REFERENCE

HAHN, E.G. (1984) Blood analysis for liver fibrosis. *Journal of Hepatology*, 1, 67.

## Nodular prurigo

MADAM, The article on nodular prurigo by Rowland Payne *et al.* (*British Journal of Dermatology*, 1985, 113, 431) suggests that half the patients studied had a metabolic cause for their pruritus. They state also that the majority had nodular prurigo in association with eczema (26 of 46 cases studied). I find it difficult to reconcile these two statements. Perhaps the authors are suggesting that all cases of eczema should be investigated metabolically: in which case the distribution of the metabolic abnormalities between the eczema and the non-eczema groups should be recorded. In fact, the only information provided is that anaemia was restricted to patients with the histology of chronic eczema. This suggests that nodular prurigo, without eczema, is not metabolically related; a curious paradox.

I wonder also whether the authors distinguished between nodular prurigo and generalized pruritus. The latter is commonly associated with metabolic abnormalities, and cutaneous signs, if present, usually consist of excoriation rather than nodules. The onset of pruritus coincides with the onset of the metabolic disorder, and is relieved when the metabolic abnormality is corrected. There is no indication from the paper whether any of these criteria were fulfilled in any of the cases studied.

Finally, the histological classification seems rather strange. I think that most pathologists would regard chronic eczema, lichen simplex and nodular prurigo as a continuum of histological changes. Criteria for distinguishing these conditions are not detailed in the paper. Yet we are told that the percentage of patients with the classical histological features of nodular prurigo was four times greater in the non-eczema than in the eczema group. If the eczema patients did not have nodular prurigo histologically, one has to question why they were included in the study.

The concluding paragraph states: 'Misconceptions about nodular prurigo abound'. I would concur with that, but fear that this study may have added to the confusion.

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## Reply

MADAM, We would like to thank Dr Russell Jones for highlighting some important aspects of nodular prurigo (NP) and we are grateful for the chance to clarify them.

The diagnosis of NP is a clinical one. Our study was restricted to patients with pruritic nodules of at least 6 months' standing. Patients were classified into two groups: (i) NP Hyde: an eruption composed almost exclusively of prurigo nodules; (ii) NP Eczema: an eczematous eruption with prurigo nodules.

The clinical and histological similarities between the two groups were striking; the differences largely reflected our arbitrary distinction between them which rested on the presence or absence of overt signs of eczema. One of our conclusions was that a continuum exists between NP Hyde and NP Eczema and that all forms of NP are intimately related to eczema.

We do not suggest that all patients with eczema should be investigated metabolically (eczema is common; eczema with nodules is rare). The distribution of metabolic abnormalities in the two groups was similar.

We agree with Dr Russell Jones' description of generalized pruritus. We did distinguish between NP and generalized pruritus. Patients without nodules were not included in our study. Incidentally, brief details were given of a patient in whom the onset of NP coincided with the onset of a metabolic disorder, and of two patients in whom NP resolved when metabolic abnormalities were corrected.

Like Dr Russell Jones, we too regard the histopathological changes of chronic eczema, lichen simplex and NP as a continuum. The microscopic appearance of 'classical nodular prurigo' was outlined. The NP Eczema patients were included in the study because they had 'an eczematous eruption with prurigo nodules'.

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J.D.Wilkinson
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M.M.Black

## *Erratum*

Dubertret, L., Chastang, C., Beylot, C., Bazex, J., Rognin, C. & Touraine, R. (1985) Maintenance treatment of psoriasis by Tigason: a double-blind randomized clinical trial. *British Journal of Dermatology*, **113**, 323.

Would readers please note that the last sentence on page 323 of the above paper was published incorrectly. This sentence should be as follows:

These results can be compared to those found in a previous unpublished multicentre 'double-blind' randomized clinical trial showing no therapeutic benefit with a tested drug (other than Tigason), but allowing us to assess the placebo effect in psoriasis.

*British Journal of Dermatology* (1986) 114, 523–526.

# *Book Reviews*

**The Urticarias.** Edited by R.H.Champion, M.W.Greaves, A.Kobza Black and R.J.Pye Edinburgh: Churchill Livingstone. Pp. 237. Price £24.00.

I find that my avidity for published reports of symposia has lessened over the years. Probably it is just a matter of ageing, but I no longer look forward so much to another dose of clotted clichés ('it should now be possible to undertake meaningful studies also in this exciting field.') and leaden, lumpy prose. Sometimes, I can see only too easily in my mind's eye the fixed expressionless faces of the original speakers, or the backs of their heads, as they drone on over their 20 slides, each with its 20 columns.

However, I have to admit that it has done me no harm at all to have been made to read through this book based on a symposium held in Cambridge on 'The Urticarias'. I have worked through solid reviews, referenced up to 1984, on mast cells, prostaglandins, anaphylatoxins, kinins and plasma protease inhibitors. I have marvelled anew at the thoroughness with which some authors investigate their patients with chronic urticaria, the cause of which can even now be established in less than 20% of cases. I found myself lost once more in the maze of food additives and depressed that the removal of azo dyes from foods in Sweden in 1978 has not yet reduced noticeably the number of patients with chronic urticaria. How tempting it must be to short circuit the whole business with an elimination diet (green tea, water, cane sugar, lamb, sea salt and whole rice) which is said to improve patients within 5–7 days if food is a causative factor. Otherwise, we are left to work our way through the splendid array of drugs described in the final section of the book.

Perhaps the answers to our questions on urticaria are no clearer than they were in 1974 when Warin and Champion published their monograph on the subject (Warin, R.P. and Champion, R.H. (1974) *Urticaria.* W.B. Saunders and Co, London), but the questions being asked are now much more complicated. The two books will sit well together in any departmental library.

J.A.Savin

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**Plant Contact Dermatitis.** Cl.Benezra, G.Ducombs, Y.Sell and J.Foussereau (1985) Ontario: B.C.Decker Inc. Pp. 353. Price £71.50.

This attractive book was designed to provide an essentially practical guide for dermatologists, general practitioners and others involved in the management of contact dermatitis due to plants.

The first 45 pages of the book give an outline of the clinical features, diagnosis (including patch testing) and treatment of plant dermatitis, together with notes on contact allergy, plant allergens and the naming of plants. Idiomatic translation has not been easy, but serious flaws seem not to have arisen.

The core of the work, which follows, deals systematically with the principal plants and woods that cause contact dermatitis. These are arranged in families alphabetically. The plant's family, 'usual' and full botanical names head the page. The text is very clearly laid out to facilitate rapid reference. Thus, each paragraph outlines a topic. One, which is essentially botanical, gives interesting features of the plant, and notes species and varieties, together with the geographical distribution, and some common names (including French and German). Another deals briefly with clinical features and offending species. Patch testing receives a variable amount of coverage concerning test substances, types of reaction, and cross-reactions. After a paragraph on miscellania, there is a list of selected references, up to and including 1983. The facing page usually has at least one excellent coloured picture of the plant, with a terse, rather cryptic, botanical description, followed by a note on the allergen and, where known, its chemical formula.

Tropical woods receive similar attention in a separate chapter, and this is followed by a brief account of miscellaneous plants. Finally, there is a neat section, based on simplified line drawings, on the terminology of plant parts, and a glossary of botanical terms. There are three indexes: (1) General, chemical and clinical names; (2) scientific names; (3) vernacular names, of plants.

The outstanding feature of this book is the large collection of excellent colour illustrations, both botanical and clinical. These put it in a class of its own. The text is not exhaustive, and the early chapters are, more or less, a primer, but, as a whole, the book contains a wealth of useful and relevant information. While it will be an invaluable practical reference book in its own right, the clinician with this book to use in conjunction with a text such as 'Mitchell and Rook' will be superbly equipped to deal with plant-related dermatoses.

J.N.S.MITCHELL

**Clinical Dermatology: A Colour Guide to Diagnosis and Therapy.** THOMAS P.HABIF. C.V. Mosby. Pp. 586. Price £55.50.

Dr Habif is adjunct Professor of Clinical Medicine (Dermatology) at the Darmouth Medical School, U.S.A. His stated audience for this book is 'practising physicians who see dermatological problems in their offices' and he aims to provide 'a practical resource for busy practising physicians'.

The text is divided into 26 chapters, 23 related to diseases or disease groups, one introductory chapter, one chapter on topical therapy and one on procedures in dermatological surgery. A 'plus point' for the book is the large number of colour illustrations, almost all of very high quality. Another positive point, particularly for the American doctor, is the very detailed account of therapy, given at the end of each disease description. Not all dermatologists will agree with the minutiae of the instructions with regard to therapy, but a general practitioner or practising physician is certainly given clear guidelines on a possible approach to therapy. As American names and trade names are used exclusively, the value of these details is somewhat diluted for a British audience.

Throughout the book there is an absence of discussion of aetiology, of mechanisms and of underlying pathology. This is perhaps understandable given the target audience, but renders it less useful for practising dermatologists or dermatologists in training.

The book should sell well to its intended readers, particularly in the U.S.A. By present day standards and given the number and quality of colour illustrations, it is good value for money.

RONA MACKIE

**Practical Genetic Counselling.** P.S.HARPER (1984) 2nd Edition. Bristol: J.Wright. Pp. 326. Price £15.00.

Dermatologists have to treat patients with a great variety of inherited diseases, yet few of us feel confident in the role of genetic counsellor. This is an important part of the management of such patients, however, and as a result of the current rapid developments in genetic engineering, this aspect of clinical practice is likely to become increasingly important in the future. Patients are becoming more knowledgeable and demanding, and the clinician who fails to offer appropriate genetic counselling, or to inform the patient that a particular test is available, may in some circumstances, be held to be professionally negligent.

Peter Harper, who is Professor of Medical Genetics in Cardiff, has performed a valuable service to the profession by producing this book, which explains the principles and processes of genetic counselling, and is aimed at the clinician rather than the geneticist. The first half of the book gives a lucid account of the inheritance of Mendelian disorders, non-Mendelian disorders and chromosomal abnormalities. The calculation of the risk in various situations is described so clearly that dermatologists with even the most tenuous grasp of mathematics will find it easy to follow. There is also a wealth of sensible and practical advice on patient management, based on the author's considerable clinical experience in this field. This includes advice on how to avoid a number of potentially embarrassing pitfalls, and how to deal with the thorny problem of 'who is to blame'. The author's discussion of the pros and cons of giving positive advice to a couple ('direction'), rather than simply providing the bare statistical facts, is also very useful. The detailed account of the construction of a family tree will be helpful to those with rare genodermatoses to report, and many nurses will be grateful to the author for pointing out to the dermatologist that the sudden arrival of a complete kindred of a dozen or more people is likely to cause consternation in the clinic.

The second half of the book describes the inheritance of diseases of various specific organs. The coverage of skin disease in this section is necessarily rather superficial, but many disorders of interest to the dermatologist (e.g. tuberous sclerosis and porphyrias) are to be found in other chapters.

This second edition of the book includes recent advances in the field, particularly in prenatal diagnosis, and there is a new chapter on recombinant DNA techniques. I now understand the Southern blot, and the value of restriction fragment length polymorphisms.

I can recommend this book to all dermatologists who are not completely *au fait* with the meaning of 45 XYt (14q 22q).

J.L.BURTON

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**Handbook of Paediatric Dermatology.** J.HARPER (1985) London: Butterworth. Pp. 204. Price £19.50.

The author is widely recognized and respected as a paediatric dermatologist and one would anticipate that a book from his pen would be authoritative, interesting, informative, and well-written and presented. Indeed, such is the case. It is very well illustrated as would be expected in a book containing 120 colour illustrations in 204 pages. Because of the large number of pictures, the text is somewhat limited, but as they are of high quality and well illustrate the points, this compensates for the text. It covers all the common skin diseases and those less common, which candidates for the Membership would be expected to have a knowledge of, such as histiocytosis X, Kawasaki Disease, graft versus host disease. There are very useful appendices at the back, including an 18 page Drug Formulary Appendix with very useful lists of local preparations, and an Appendix of Useful Textbooks for Parents, and useful addresses both in the United Kingdom and the U.S.A. for such organizations as the National Eczema Society. Important points in the text, which is very well written, are highlighted with comments in dark print in the margin or a blue box in the text, e.g. the causes of urticaria or erythema nodosum. Every chapter ends with a number of references for further reading.

The book is said to be 'designed for paediatricians, dermatologists or any physician who has to deal with children suffering from skin disorders'. I would think most dermatologists would find this text a little superficial to have as a reference book on their shelves, but I recommend it very strongly to those commencing dermatology, and it would be particularly useful to paediatricians. Candidates for the Membership would find it especially useful as a quick review, not only on paediatric dermatology but on dermatology in general.

D.BURROWS

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**Contact Allergy: Predictive Tests in Guinea-Pigs (Current Problems in Dermatology, Volume 14).** Edited by K.E.ANDERSEN and H.I.MAIBACH (1985) Basel: Karger. Pp. 229. Price £50.70.

In this book, proponents of the currently available methods of testing for the sensitization potential of chemicals in guinea-pigs describe and discuss each method, together with some of the data generated. In most instances the originators of the technique are the authors and, when this is not the case, the authors are experienced users of the technique. At the end of the book, the editors review all thirteen of the methods, giving much-needed guidance on the choice of test, to which, as might be expected, there is no simple answer. The book opens with a thorough introductory chapter on the epidemiology of contact sensitization.

An excellent book that already goes part of the way along this same road is Thomas Maurer's manual of predictive test methods, *Contact and Photocontact Allergens* (volume 3 in the Marcel Dekker Dermatology series), but, as a contributor to this new and larger book, he would, I think, agree that its coverage is in greater depth than his. Maurer's book, however, also covers photocontact sensitization, which this book does not.

Dermatologists and others with anything more than a strictly clinical interest in contact dermatitis, will find Andersen and Maibach's book to be the most useful review currently available of guinea-pig testing with potential contact sensitizers.

R.J.G.RYCROFT

**Psoriasis.** Edited by H.H.ROENIGK, JR. and H.I.MAIBACH (1985) New York: Marcel Dekker. Pp. 667. Price $126.00.

Psoriasis is a multiauthor volume which is number 5 in a series of specialist dermatology books. Seventy-three contributors from the U.S.A. and Europe have produced a very readable book that can be divided into three sections under the headings: clinical features, pathogenesis and treatment.

The clinical section enlightens us on lesser known clinical features. For example, Epstein and Maibach's chapter 'Eczematous psoriasis' will strike chords in the experienced clinician and Harvey Baker writes informatively on pustular psoriasis. There are chapters on the eye, the gut, arthritis, cardiovascular complications, (occlusive vascular episodes are said to be more prevalent), the scalp and nails, the Koebner response, psychological aspects and epidemiology.

In the section on pathogenesis Weinstein summarizes the data on cell proliferation kinetics and there are also chapters on polyamines, arachidonic acid, leukotrienes, proteinases, cyclic nucleotides, immunology and disordered neutrophil function.

More than half of the 665 pages are taken up by a very comprehensive review of psoriasis treatment. Although many British dermatologists would agree with Richard Stoughton when he refers to topical corticosteroids as the 'wonder drugs' of therapy they would certainly not share his enthusiasm for their use in psoriasis. However, in a short chapter on systemic corticosteroid use, Lahti and Maibach give a very responsible and cautious account of their use. Photochemotherapy and retinoids are fully covered, as are retinoid therapy singly or in combination (REPUVA). There are even chapters on dialysis and plasma exchange.

One criticism might be that many of the black and white clinical photographs are poorly reproduced and a few are so indistinct as to merit exclusion. This is a pity because it threatens to spoil an otherwise excellent book which will be of special interest to the clinician treating psoriatic patients and to dermatologists in training. The price is high but the book should be a welcome addition to the shelves of many dermatological departments.

P.G.GOODWIN

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| milligram(s) ($10^{-3}$g) | mg | second(s) | s | gravitational acceleration | *g* |
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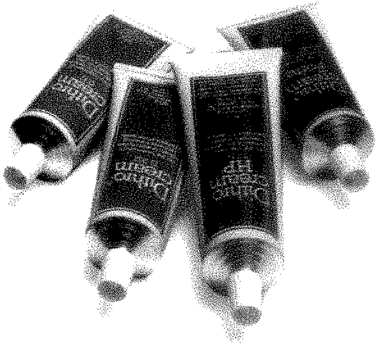

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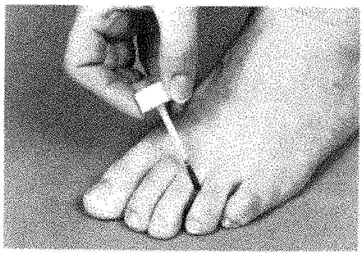

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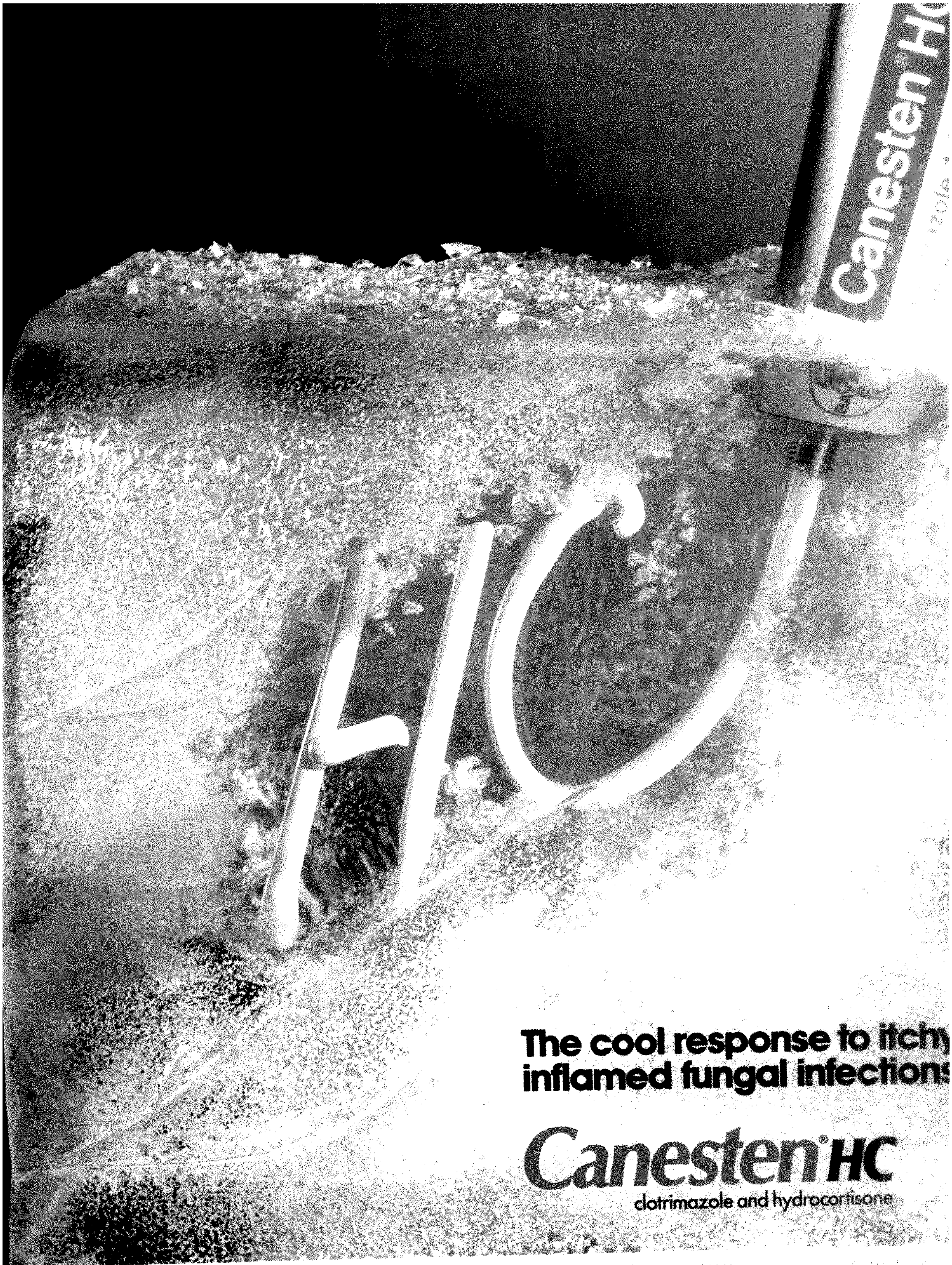
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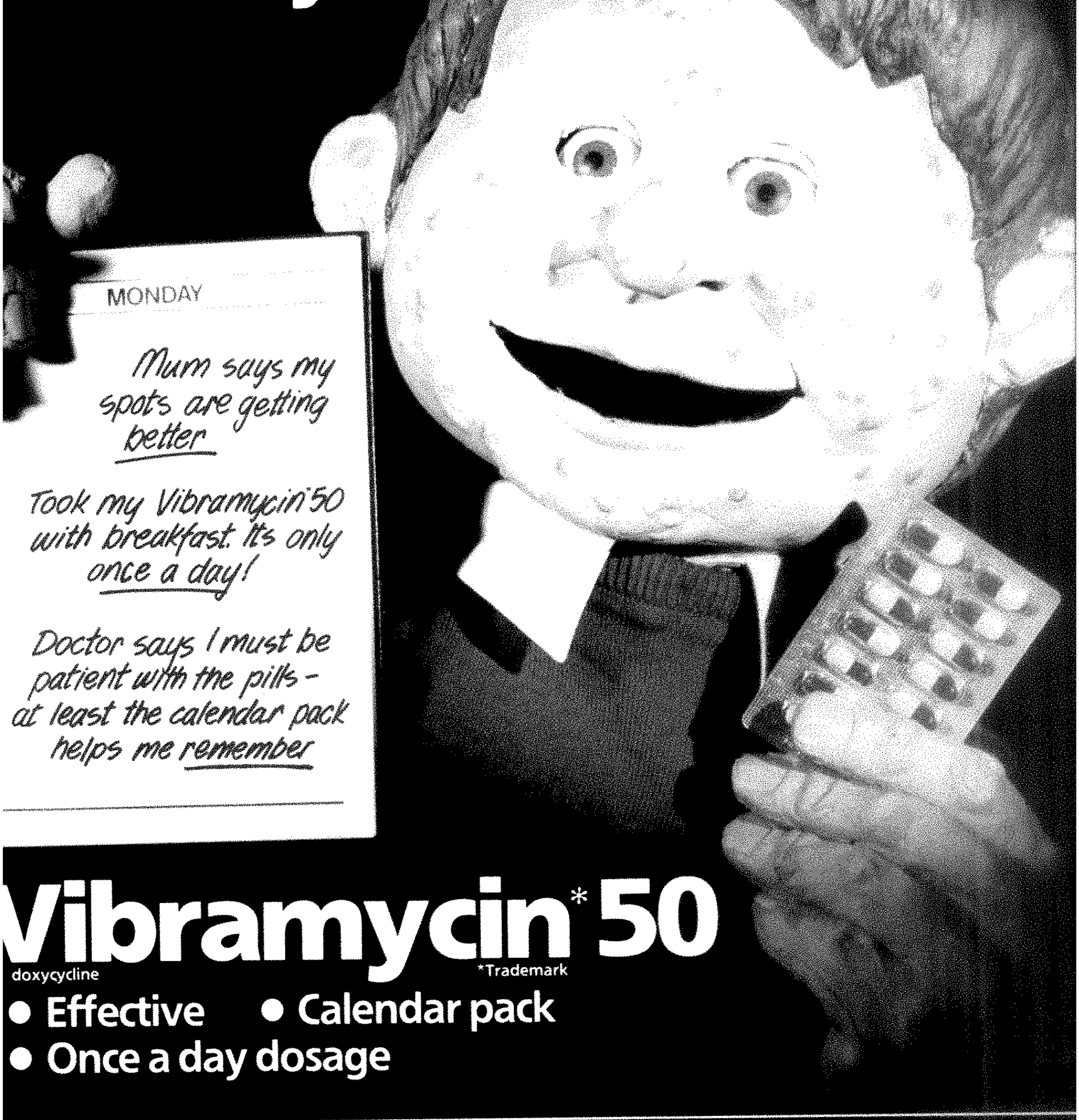
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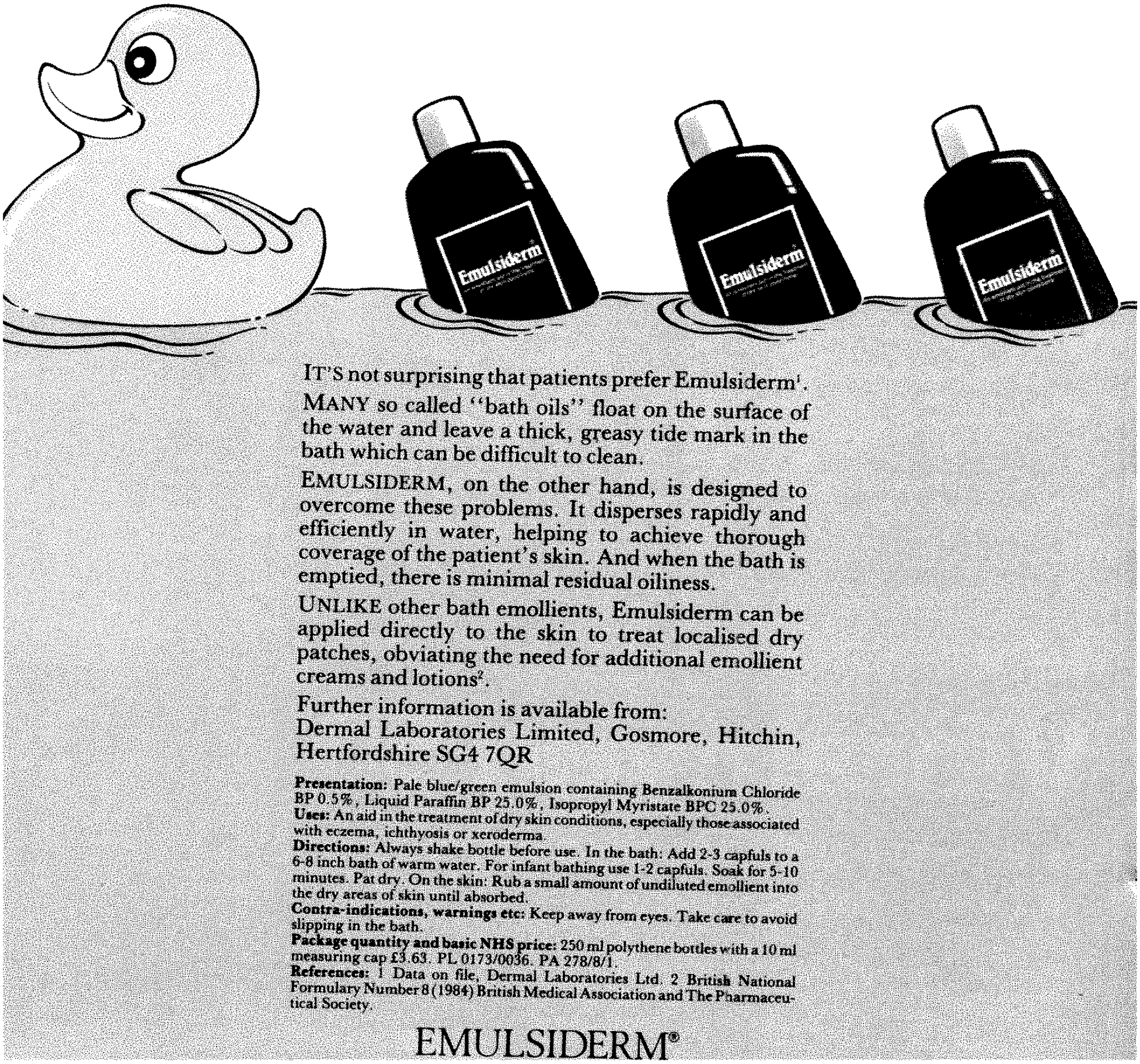

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*British Journal of Dermatology* (1986) 114, 527–533.

***Comment***

# The treatment of Raynaud's phenomenon

PAULINE M.DOWD
The Middlesex Hospital, Mortimer Street, London W1N 8AA, U.K.

Although the cause of Raynaud's phenomenon (RP) is not yet known, a pathophysiological role has recently been suggested for pharmacological abnormalities of the constituents of the blood, which are reported in a proportion of patients with both primary and secondary RP. Hypersensitivity of the digital cutaneous vasculature to the vasoconstrictor actions of serotonin, and defective production of vasoactive mediators may be of pathophysiological significance. Circulating cryoglobulins, cryoagglutinins, *in vitro* platelet activation with elevated levels of circulating beta thromboglobulin, and reduced red blood cell deformability have all been considered to contribute to the local ischaemia in the microvasculature. For this reason, several of the newer treatments employed in the medical management of RP modify these reported abnormalities.

There are four large groups into which the existing therapies for RP can be divided—namely, conservative therapy, sympathectomy, drug therapy and plasmapheresis.

### *Conservative therapy*

Conservative therapy may be all that is required in mild RP. Patients should be advised to stop smoking and to avoid cold temperatures, especially sudden changes of temperature and drafts. They should protect the hands and, if necessary, the feet by wearing warm gloves and socks. Electrically-heated gloves and socks are now available and can be extremely useful in the management of uncomplicated Raynaud's phenomenon of moderate or severe degree.

### *Sympathectomy*

There are anecdotal reports of the efficiency of chemical sympathectomy by procaine stellate blockade in patients with Raynaud's phenomenon severe enough to cause digital ulceration. Immediate clinical improvement in blood flow may result, raising the skin temperature of a digit to the normal vasodilatation level. The procedure can, if necessary, be performed on an hour to hour basis in acute vascular insufficiency resulting from Raynaud's phenomenon, whilst long-term therapies are instituted.

Results of surgical sympathectomy in functional vasospasm of the upper limbs are disappointing, mainly due to high local reactivity of the blood vessels and the early return of autonomous vascular tone. Relapse after sympathectomy may develop at any time after operation and the hands may be uncomfortably dry as a result of sudomotor denervation. Long-term follow-up has demonstrated a lack of benefit in patients who either had a collagen disease at the time of operation, or developed one subsequently (de Takats & Fowler, 1962). For these reasons, surgical sympathectomy has fallen into disrepute in the management of Raynaud's phenomenon.

### *Drug therapy*

Drug therapy is indicated for patients who have severe symptomatic primary or secondary RP refractory to conservative measures, or who experience degrees of digital ischaemia sufficient to cause tissue infarction.

Drugs affecting the sympathetic nervous system have been extensively used, but reports of their efficiency are mainly anecdotal, and there is a dearth of adequately-controlled trials. Recently, attention has been centred on the use of selective $\text{alpha}_1$ adrenoceptor blocking agents. Drugs with both $\text{alpha}_1$ and $\text{alpha}_2$ receptor blocking activity, adrenergic neuronal blocking agents and drugs acting centrally, such as methyldopa, have also been studied.

The alpha adrenoceptor blocking agent, phenoxybenzamine (10–80 mg daily) provides symptomatic relief in some patients (Gifford, 1963). Tolazoline, a weaker alpha blocking agent which has a direct vasodilator effect on vascular smooth muscle, may, in large doses, produce an improvement in skin blood flow in vasoconstricted normal subjects, and has been found occasionally to be useful in RP in combination with other agents at a dose of 25–50 mg qid (Coffman & Cohen, 1971). The frequency and severity of side-effects precludes these agents from being used routinely in the management of RP, and they are contra-indicated in patients who have an underlying peptic ulcer or cardiac disease.

Prazosin hydrochoride and indoramin have selective activity for post-synaptic $\text{alpha}_1$ receptors, with apparently little effect on $\text{alpha}_2$ receptors; $\text{alpha}_2$ autoregulation still can occur and relatively little tachycardia results. In an uncontrolled trial, clinical improvement was reported in 50% of patients with moderate to severe RP receiving prazosin 2–20 mg daily in divided doses (Waldo, 1979). Indoramin 30 mg tid resulted in a significant reduction in the number of attacks of RP in a double blind trial (Robson *et al.*, 1978) with a concomitant increase in blood flow measured by digital venous plethysmography.

Guanethidine is an adrenergic neurone blocking agent which interferes with release of noradrenaline at the neuro-effector junction, and has been shown by xenon washout studies to increase finger blood flow after cooling in patients with RP at doses of 30–50 mg or more daily (Kontos & Wasserman, 1969).

Methyldopa acts both on the CNS and as a false neuro-transmitter for noradrenaline. In uncontrolled studies it improved digital temperature and reduced the severity of vasospasm in RP when given orally at 1–2 g daily in divided doses (Varadi & Lawrence, 1969; Hansteen, 1976).

Reserpine, an adrenergic neurone blocking agent, depletes sympathetic nerve endings of noradrenaline, and is effective orally and by intra-arterial injection. Oral reserpine has been shown to attenuate adrenergic vasoconstriction in response to cold. Intra-arterial reserpine has a short vasodilator action, unrelated to noradrenaline depletion. Oral reserpine (0·25–1 mg daily) produces amelioration of symptoms in about 50% of patients. Solitary intra-arterial injections resulted in subjective and objective improvements in RP ranging from 1 day to 6 weeks in duration, with a mean of 18 days (Willerson *et al.*, 1970). Nilsen and Jayson (1980) reported clinical improvement lasting for up to 5 months in seven patients with systemic sclerosis and severe RP. This was accompanied by an increase in basal skin blood flow and loss of excessive reduction in blood flow after local cooling, as assessed by clearance of epicutaneously-applied $\text{xenon}^{133}$ in controlled environmental conditions. If reserpine was re-injected as soon as the response to cooling became abnormal again, ischaemic ulcers apparently healed faster and relapses could be avoided. Postural hypotension tends to limit long-term oral therapy with guanethidine and methyldopa, and a high incidence of depression is an added problem with reserpine.

Topical nitrates, i.e. nitroglycerine 2% ointment, have been reported to improve digital temperature and blood flow, and may provide symptomatic improvement for patients with RP (Beaucher, 1979). However, benefits are temporary, frequent re-applications are required, and experiences as to its efficacy differ (Coffman, 1979). Ischaemic cardiac injury has been reported in industrial workers abruptly withdrawn from chronic exposure to nitrates (Lange *et al.*, 1972). It would seem wise, therefore, to restrict the use of topical nitrates to those patients who have severe Raynaud's phenomenon unresponsive to other medical treatment and who are not expected to discontinue the drugs abruptly during the warmer seasons. The zealous use of this topical therapy by enthusiastic patients should be discouraged.

Nicotinic acid (niacin) and nicotinyl alcohol may increase hand and forearm blood flow, but studies have not shown a consistent increase in skin or muscle blood flow. Long-term studies (Coffman & Mannick, 1972; Hansteen & Lorentsen, 1974) do not provide a basis for the use of these drugs in peripheral vascular disease of the upper or lower limb. Inositol nicotinate (Hexopal) is a long-acting vasodilator acting by slow release of nicotinic acid. Both its vasodilator and fibrinolytic properties have provided a rationale for its use in Raynaud's phenomenon (Ring & Bacon, 1977; Sommer, 1965). Significant, but short-lived, increases in the basal temperature of the hands were noted within one week of the commencement of inositol nicotinate therapy in six patients with Raynaud's phenomenon and RA. Personal experience with this drug has been disappointing.

Forrest (1978) and Judge (1978) reported beneficial effects of naftidrofuryl (Praxilene) and Nilsen (1979) reported increased mean basal blood flow and improvement in cooling tests by xenon$^{133}$ clearance during naftidrofuryl treatment of patients with primary and secondary Raynaud's phenomenon. No description of clinical responses in relation to therapy was given in the latter study.

An anecdotal report of alleviation of vasospasm in a 7-year-old girl with Raynaud's disease treated with griseofulvin (Creery *et al.*, 1968) was followed by several reports of successful treatment of Raynaud's phenomenon with griseofulvin in doses of 0·5–2·0 g daily (Charles & Carmick, 1970; Allen, 1971; Sabri *et al.*, 1973). Improvement was more marked in those patients receiving therapy for Raynaud's disease as opposed to RP associated with systemic sclerosis. The mode of action would appear to be a direct effect on vascular tone. Long-term studies to establish whether griseofulvin offers a lasting clinical benefit in Raynaud's phenomenon are lacking.

Anabolic hormones such as stanozolol have been used in treating Raynaud's phenomenon in the light of their ability to enhance fibrinolytic activity and lower blood viscosity. Increased fibrinolysis has also been shown to occur after infusion of low molecular weight dextran (LMWD). There have been several reports of the beneficial effect of LMWD infusions in patients with Raynaud's phenomenon (Holti, 1965; Burrows, Shanks & Stevenson, 1968; Fountain & Stevens, 1966; Alani, 1970; Wong *et al.*, 1974). As well as increasing fibrinolysis, LMWD is an effective plasma expander, reducing red blood cell sludging in capillaries, supposedly by coating erythrocytes and vessel walls, and thereby reducing frictional resistance. By coating platelets and red cells, LMWD reduces their adhesiveness and has an antithrombotic effect. For these reasons, LMWD would tend to promote increased blood flow and tissue perfusion. Of the studies reporting favourable responses to the use of LMWD in RP, only that of Wong *et al.* (1974) examined clinical improvement in relation to measurements of cutaneous blood flow. The other studies of LMWD in RP were uncontrolled anecdotal studies, lacking objective measurements.

### *Newer therapeutic agents*

These include the prostaglandins, calcium channel blocking agents, the serotonin antagonist ketanserin, and the angiotensin-converting enzyme inhibitor, captopril.

Prostaglandin $E_1$ and prostaglandin $I_2$ are powerful vasodilators and inhibitors of platelet aggregation. In controlled studies, 72 h central venous infusions of prostaglandin $E_1$ (4–12 ng/kg/min) (Martin *et al.*, 1980, 1981; Clifford *et al.*, 1980) resulted in a decrease in the frequency, severity and duration of attacks of Raynaud's phenomenon, improvement in pain and cold tolerance, and healing of ischaemic digital ulceration. Subjective benefits were accompanied by increased finger and hand temperatures, assessed by quantified infra-red thermography, and improvement in blood flow to the hand, as measured by Doppler-derived radial artery pulsatility, finger pulse volume recordings and radiometry. However, in further studies it was not possible to substantiate that a sustained significant increase in finger temperature occurred in association with symptomatic improvement (Dowd, 1984). Intravenous infusions of prostaglandin $I_2$ ($PGI_2$, prostacyclin, epoprostenol, flowlan) for 72 h at doses of 2·5–10 ng/kg/min in open studies (Dowd *et al.*, 1981) resulted in a decrease in the frequency, severity and duration of attacks of RP lasting for several weeks in the majority of patients. Cold tolerance, pain and stiffness of the hands were similarly improved, and a sustained increase in finger temperature, measured by thermography, was noted two weeks after completion of the infusion. Inhibition of platelet aggregation (reported to be enhanced in RP) occurred during the infusion in those patients who received the higher doses of $PGI_2$, but this was not sustained for any substantial length of time after termination of the infusion. Red blood cell deformability, which has been shown to be decreased in RP and especially in RP occurring in association with systemic sclerosis, was increased by infusion of both PGE and $PGI_2$. Increased red cell deformability, however, was only evident two weeks after infusion of $PGI_2$ in less than 50% of patients. The use of $PGI_2$ also proved beneficial in placebo-controlled intermittent intravenous infusions, given over 5 days (Belch *et al.*, 1981, 1983). The unexpected longevity of the response to infusions of $PGI_2$, and its mode of action in producing a sustained clinical improvement in RP, remain unexplained. Side-effects of $PGI_2$, in particular hypotension, are troublesome and may necessitate termination of an infusion. The use of stable analogues of prostacyclin, given orally or epicutaneously, merits further investigation.

The slow calcium channel antagonists are an important group of agents which block cellular uptake of calcium. Since smooth muscle contractility depends upon calcium influx into the cell, inhibition of calcium influx reduces contractility and can lead to muscle relaxation. Nifedipine and verapamil are of greatest current interest, and nifedipine has emerged as a potent vasodilator with effects on both the coronary and peripheral arterial beds. There are now numerous reports of favourable clinical responses to oral nifedipine 20–80 mg orally/day in primary and secondary RP in placebo-controlled studies substantiated by objective measurements of blood flow (Kahan *et al.*, 1981, 1982, 1983; Smith & McKendry, 1982; Pierce, Della Valle & Wielenga, 1983; Rodeheffer *et al.*, 1983; Creager *et al.*, Sauza *et al.*, 1984; Nilsson *et al.*, 1984; Mikkelsen, Andersson & Lederballe Pedersen, 1978; Winston *et al.*, 1983; Yamada *et al.*, 1984). Flushing, light-headedness and ankle swelling are the commonest side-effects, frequently, but not invariably, dose-related. They may disappear with continued therapy, and are much less marked with the use of the newer slow-release preparation (personal observation). Therapeutic benefit is seen in about 60% of patients with RP treated with nifedipine. The numerous reports substantiating Kahan's original observation make nifedipine one of the most useful agents currently available for the treatment of Raynaud's phenomenon.

It has been suggested that the digital cutaneous vasculature in patients with RP may be hypersensitive to 5-hydroxytryptamine (5HT, serotonin). The selective $5HT_2$ receptor blocking agent ketanserin has therefore been investigated in RP (Stranden, Roald & Krohg, 1982). Digital blood flow and skin temperature increased significantly after ketanserin injection. Unfortunately, although the authors reported symptomatic relief with oral ketanserin (20 mg bd), the study did not report any long-term clinical subjective or objective benefit after *i.v.* ketanserin. A double-blind crossover study of oral ketanserin 40 mg tid in RP associated with connective tissue diseases (Roald & Seem, 1984) showed clinical improvement in 8 out of 10 patients on ketanserin and none on placebo, and a reduction in recovery time after cold provocation. Orally-administered ketanserin may also prove, therefore, to be an effective treatment for RP associated with connective tissue disorders, and merits further study.

Captopril, a serum angiotensin converting enzyme inhibitor, when administered intravenously, resulted in appreciable improvement in the digital circulation subjectively, objectively and plethysmographically during monitoring in a patient with apparently primary RP. Subsequently, oral captopril 37·5 mg daily resulted in remarkable improvement over several months of therapy. Pharmacological studies employing serine proteinase inhibitors and an angiotensin II antagonist suggested that the vasodilator action of captopril might be caused mainly by accumulated circulating kinins, and not by the inhibited production of angiotensin II. More extensive studies are required to confirm the promising effects of captopril in the relief of RP.

### *Plasmapheresis*

Plasma exchange with exhausted plasma or a combination of fresh frozen plasma and purified protein fraction, weekly for 4–5 weeks, has resulted in an increase in blood flow to the hand, an increase in vessel patency and clinical improvement, lasting for periods of 4 weeks to 6 months after treatment in both open and controlled studies (Talpos, Horrocks & White, *et al.*, 1978; Goyle & Dormandy, 1976; O'Reilly *et al.*, 1979). Reduction of blood viscosity, red blood cell deformability, decrease in rate and extent of platelet aggregation, reduction in plasma viscosity and beta thromboglobulin, and reduction in plasma fibrinogen concentrations have all been reported after plasmapheresis, but its mode of action remains, as yet, imperfectly explained. The limited availability of blood processors, possible adverse haemodynamic effects and expense limit the usefulness of this treatment in clinical practice.

Almost all the published results of treatment of RP have included patients with RP secondary to a variety of causes, and have also included patients with primary RP. This heterogeneity of patient selection makes it difficult to interpret the results of any therapy in relation to the presence or absence of associated disease. In order to assess adequately the results of new therapies, it is essential that future studies be carried out in homogeneous and carefully documented groups of patients, whose response to therapy might be expected to be more consistent than the responses from patients with RP due to a variety of causes.

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**Laboratory and Clinical Investigations**

# A possible role for squalene in the pathogenesis of acne. I. *In vitro* study of squalene oxidation

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## SUMMARY

An *in vitro* study of the oxidation of squalene, and a description of factors acting on this transformation are presented. Thin layer chromatography was used to quantify the products generated by different oxidation processes. The results clearly show that squalene is a highly effective oxygen-scavenging agent. Its oxidation may first induce comedogenesis and, as a secondary event, cause a large reduction in oxygen tension in the human pilo-sebaceous duct. Porphyrins were confirmed to be highly efficient catalytic factors in the squalene oxidation process. The relationships between comedogenesis, bacterial colonization, and the role of sebum in the pathogenesis of acne are discussed in the light of these findings.

Of the human skin surface lipids (SSL), squalene is known to have a purely sebaceous origin. It has been used, therefore, as a marker in numerous studies dealing with human sebaceous gland physiology, which have recently been reviewed by Agache and Blanc (1983). This unsaturated hydrocarbon ($C_{30}H_{50}$), initially called spinacene, (Chapman, 1923) can be isolated in large amounts from the liver of the family *Squalidae* (sharks).

From a biochemical point of view, squalene is a key molecule in cholesterol ring biosynthesis and, consequently, in the steroid pathway. Transformation of squalene to the sterol ring is a rapid reaction in the liver of most animal species, which is the reason why in such species (sharks excepted) this molecule is found only in trace amounts in the liver (Deuel, 1955).

From a chemical viewpoint, squalene is a molecule highly susceptible to oxidative phenomena, due to the presence of six carbon double bonds which bind atmospheric oxygen (Imaeda, Ohsawa & Yokota, 1983; Golub, Rosenberg & Gemmer, 1977; Chapman, 1923).

The continual presence of some 10–15% squalene in human SSL, appears to be a biochemical

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curiosity, common to few animal species (Lindholm *et al.*, 1981). From a dermatological standpoint, oxidized squalene has been described as cytotoxic, irritant and strongly comedogenic (Mills, Porte & Kligman, 1978; Yamamoto *et al.*, 1982; Motoyoshi, 1983). The ability of squalene oxides to induce comedogenesis has been shown to be correlated with the amount of bound oxygen per molecule (Motoyoshi, 1983). However, there is little analytical description of squalene oxidation in the literature, and there have been few studies of the oxidation kinetics and its effectors, or quantification of the compounds generated by this process (Kaplan & Kelleher, 1972; Leong, Sam & Toh, 1976).

The aim of the first phase of the present study was to investigate the general characteristics of the *in vitro* oxidation of squalene, and to explore some of the factors which may influence this reaction. Particular emphasis was placed on the description and quantification of squalene oxides, as a baseline for the second phase of the study, concerned with their distribution, *in vivo*, in acne patients, in SSL or in acne lesions. Occurrence of these oxides in acne lesions was first described by Nicolaides *et al.* (1970).

## METHODS

### *Irradiation procedures*

Irradiation was carried out by exposing the different materials to an Osram-Vitalux lamp (300 watts), emitting UV and visible radiation. Preparations were placed in the axis of the lamp, at a distance of 0·45 m, such that the temperature reached 31°C ± 1°C, with UV-A and UV-B equal to 0·03 mW/cm$^2$ and 0·5 mW/cm$^2$, respectively.

In each experiment, 18 $\mu$g of pure squalene (Fluka, puriss. grade) was deposited on small Teflon platelets (6 mm diameter) by gently pouring 5 $\mu$l of a 3·6 mg/ml solution in chloroform over the surface of the platelet. The theoretical distribution density was therefore about 60–64 $\mu$g/cm$^2$, a value comparable to that found on the forehead of a normal adult.

*Ambient oxidation.* A group of platelets was left on the laboratory bench, at ambient temperature (22°C ± 2°C) under standard lighting conditions, for a maximum of 7 days. Each day, two platelets were taken for analysis of their content of pure squalene by thin layer chromatography (TLC).

*Oxidation under UV and visible irradiation.* Groups of platelets were irradiated for various periods of time and the content of pure squalene then determined by TLC. A group of platelets was included in which 18 ng of pure protoporphyrin dimethyl-ester (PP) (Sigma) was added to the 18 $\mu$g of squalene in the initial chloroform solution, giving a ratio of 1 part PP to 1000 parts squalene.

### *Analytical methods*

*Thin layer chromatography.* After irradiation, the Teflon platelets were immediately pressed against the pre-adsorbent area of high performance TLC plates (Whatman LHP.K) by means of a stainless steel stirrup, according to a technique previously described (Saint Leger & Bague, 1981). The TLC plate was then run in four successive solvent migration baths (all solvents were analar grade):

(1) hexane (run up to 10 mm from the top);
(2) hexane-ether (90:10), (run up to 100 mm from the top);

(3) hexane-ether-acetic acid (70:30:1) (up to 35 mm from the top);

(4) chloroform-methanol (50:50), for a 1 cm run, to ensure migration of the most polar compound derived from squalene oxidation, referred to as compound D (Fig. 1). This compound does not normally migrate in the first three baths.

After drying, the TLC plates were dipped into a 3% aqueous sulphuric acid solution for 30 s and charred at 170°C for 30 min. The intensity of the spots was then measured by photodensitometry (using a Shimadzu CS 920). External standards of pure squalene (18 μg) were run at each side of the TLC plate, to calibrate the photodensitometric readings, which increased linearly with increasing amounts of squalene (Fig. 2). Four other spots appeared (Fig. 1), and these are referred to as compounds, A, B, C, and D, apparently the major products of squalene oxidation.

*Preparative extraction of compound D.* This was carried out to allow a determination of the relation between amounts of compound D and photodensitometric readings so that this relation could be compared with that of pure squalene.

One gram of pure squalene was poured into a large glass basin with a surface area of 700 cm$^2$, and spread to form a uniform layer. This was placed in the lamp axis, and irradiated for 48 h. The material was then dissolved in 200 ml of a 2:1 chloroform-methanol mixture and concentrated under vacuum, to approximately 20 ml. Freshly washed silica gel (Merck-Kiesegel 50) was added to completely absorb the solution, and then poured into a glass column.

Successive washings of the column with hexane-ether (80:20) eliminated neutral compounds (squalene) and part of the polar squalene oxides. A final wash of the column with 2:1 chloroform-methanol desorbed the most polar lipid, D, the degree of purity of which was then measured by TLC. The solution was finally evaporated under vacuum, and the residue, D, was weighted and dissolved in 2:1 chloroform-methanol to yield a solution containing 10 mg/ml.

Compound D was submitted to the following tests: elemental analysis (proportions of C, H, and O), determination of nuclear magnetic resonance (NMR) spectrum, viscosity measurement,

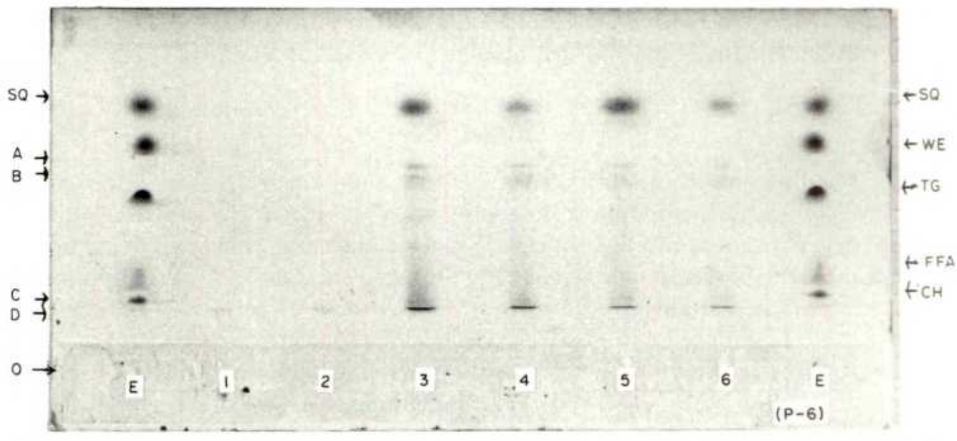

FIGURE 1. Chromatographic profile of squalene and its derivatives produced on irradiation (compounds A, B, C and D): lanes 1 and 2: 36 and 18 μg of squalene, respectively, irradiated for 22 h; lanes 3 and 4: 36 and 18 μg of squalene, respectively, irradiated for 5 h; lanes 5 and 6: 36 and 18 μg of squalene, respectively, irradiated for $2\frac{1}{2}$ h. E = external standards (SQ, squalene; WE, wax esters; TG, triglycerides; FFA, free fatty acids; CH, cholesterol); O = origin.

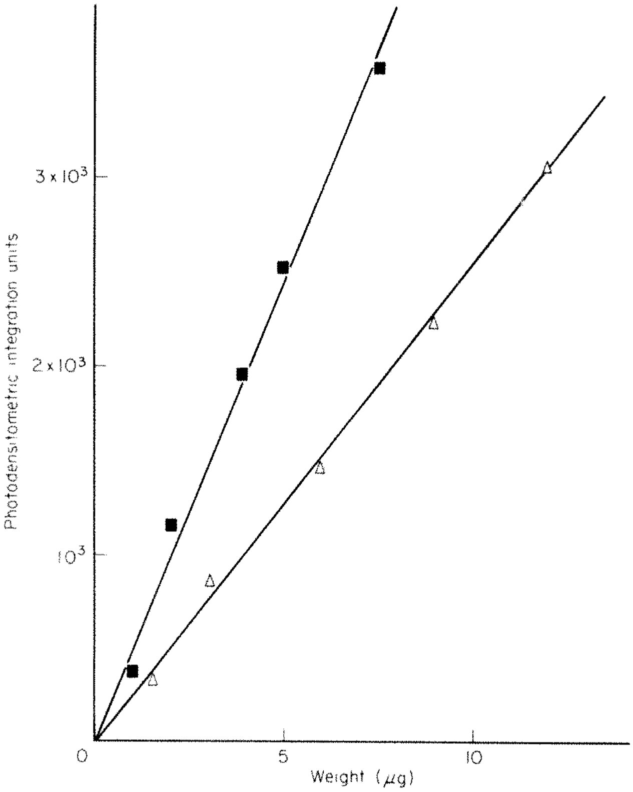

FIGURE 2. Photodensitometry of pure squalene (△) and compound D (■).

assessment of comedogenic activity by topical application to rabbit ears using the method of Kligman, Wheatley & Mills, (1970). Pure squalene was used as a control.

## RESULTS

Figure 3, a and b, illustrates the kinetics of squalene degradation under standard room lighting, and under UV and visible irradiation from a lamp with or without the presence of porphyrin. Squalene was degraded more rapidly under UV and visible irradiation from the lamp (Fig. 3b) than under standard laboratory conditions (Fig. 3a) and the rate of degradation was further increased in the presence of porphyrin.

Squalene oxidation, with or without porphyrin, yielded four major compounds referred to as A, B, C and D (Fig. 1), the amounts of which increased linearly with time of irradiation for exposure periods of up to $3\frac{1}{2}$ hours (Fig. 4). Over longer periods, compounds A, B and C tended to disappear, while the concentration of D increased considerably, suggesting that A, B and C are intermediate compounds generated during the squalene oxidation process, while D is the final reaction product. However, when small amounts (μg) of squalene were irradiated for a long period (20–22 h), no oxidation products could be detected (Fig. 1). Compound C has the same $R_f$ value as that of cholesterol. It was found that the relationship between quantity and

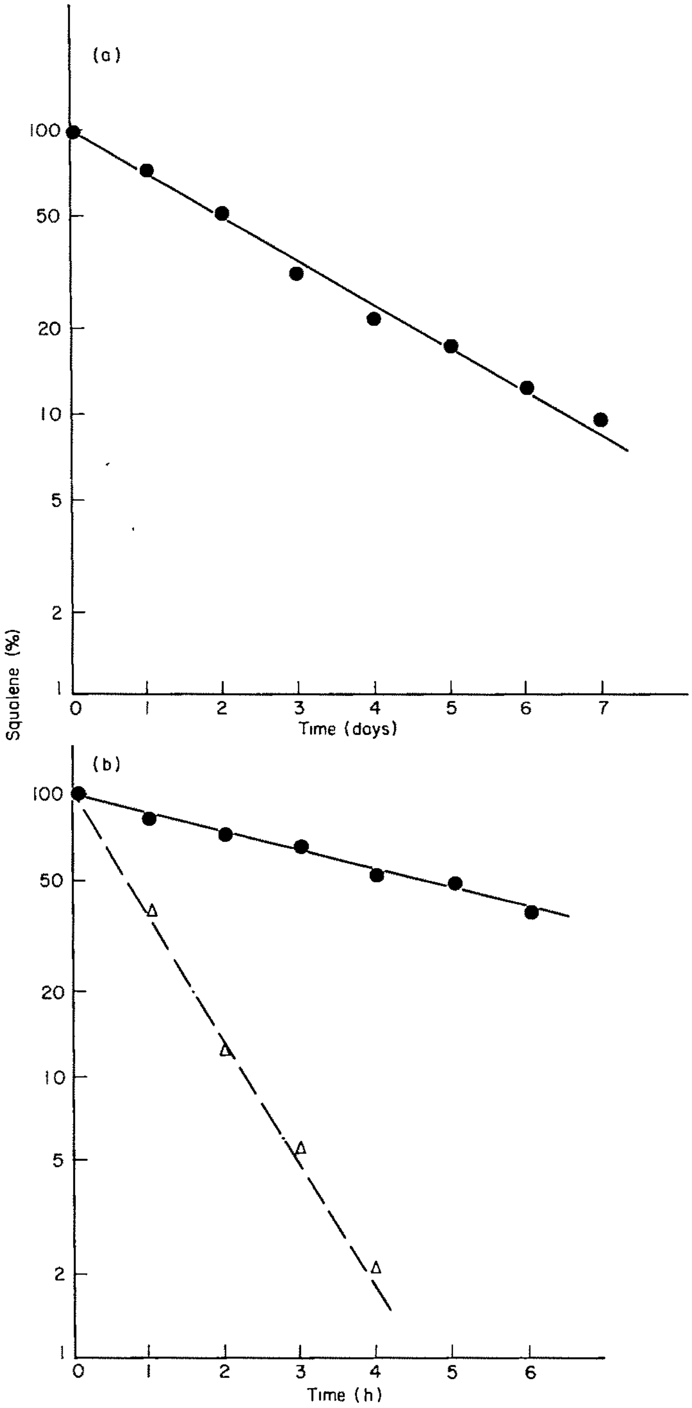

FIGURE 3. Kinetics of oxidation of pure squalene (amount of squalene expressed as a percentage of the initial amount present): (a) under ambient laboratory conditions; (b) under UV + visible irradiation (●——●), and under UV and visible irradiation and in the presence of 1 part per 1000 protoprophyrin dimethyl-ester (△-·-△).

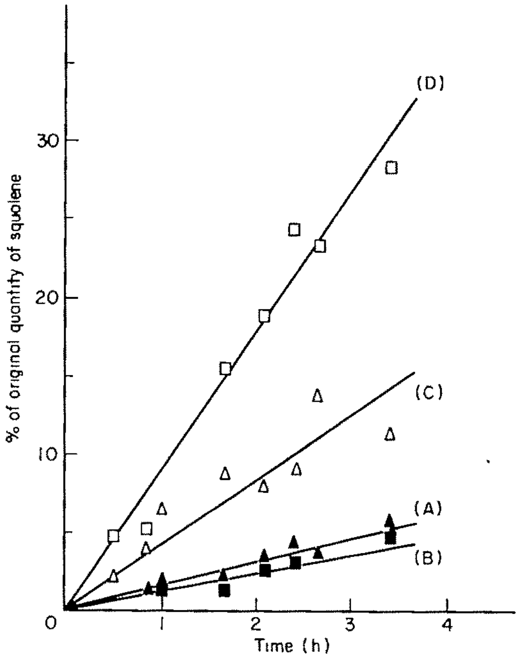

FIGURE 4. Appearance of degradation products A, B, C and D, on exposure of squalene to UV and visible radiation.

photodensitometric readings for compound D was directly correlated with that for squalene (Fig. 2). The ratio:

$$\frac{A \text{ comp. D}}{A \text{ squalene}} = 0{\cdot}52 \pm 0{\cdot}02 \text{ (mean} \pm \text{SE)}$$

where $A = \mu g$ of compound D or squalene/photodensitometric unit.

When porphyrin was added to squalene, under the same conditions of irradiation, a similar pattern of lipids was obtained, with an equal yield of compound D (35–40%).

Elemental analysis of compound D revealed a composition of carbon, 65·6%, hydrogen, 8·6%, and oxygen, 25·8%, while pure squalene has the composition: carbon, 87·7%, hydrogen, 12·3%. This confirms the ability of squalene to bind large amounts of oxygen.

Compound D appeared as a yellowish and highly viscous material (viscosity 26·2 cp at 30°C; squalene has a viscosity of 11·5 cp at same temperature), and its NMR spectrum revealed a polymeric form of the squalene unit in which the double-bonds had been substituted by aldehyde, ketone and hydroxyl groups. This is in agreement with the findings of Furhop, Baccouche & Penzlin (1980).

Application of compound D to rabbit ears confirmed its ability to induce comedones, in contrast to pure non-oxidized squalene, which did not.

## DISCUSSION

Under normal conditions of light and temperature, pure squalene tends to be degraded into polar lipids. In our experimental conditions, some 90% of the squalene initially present, either alone or included in an artificial mixture of SSL, disappeared during a 6-day period. Under UV and visible radiation, and also in the presence of porphyrin, the kinetics of transformation appeared much more rapid, and, in all cases, products with the same chromatographic profile were produced, implying that the action of porphyrin is as a catalytic agent which modifies the kinetic constant of the reaction by producing a rapid supply of singlet oxygen with which squalene preferentially reacts (Kaplan & Kelleher, 1972; Leong *et al.*, 1976).

From a practical point of view, the fact that the $R_f$ value of compound C is identical to that of cholesterol may lead to inaccuracy when analysing the cholesterol content of oxidized SSL.

When in a completely oxidized form, the squalene was found to have absorbed a large amount of oxygen, one quarter of its molecular weight, a value very similar to that obtained by Chapman (1923). This, in itself, is of considerable importance with respect to the *in vivo* situation in the human pilo-sebaceous ducts. The approximate dimensions of a pilo-sebaceous unit give an estimate of its maximum volume of about $10^{-5}$ ml. Therefore, 40 ng of squalene, when completely oxidized, would be sufficient to deplete the oxygen tension in the follicle, where microgram quantities of pure squalene can be found (Downing, Stewart & Strauss, 1981).

Thus, squalene oxidation appears to be a process which can produce micro-aerophilic conditions, and so set up an ecological niche for anaerobic flora such as propionibacteria. Bacterial colonization appears to be a secondary effect of comedogenesis induced by formation of squalene oxides.

From a bacteriological point of view, the porphyrins have been clearly shown to be synthesized *in vivo* by *Propionibacterium spp.* (Cornelius & Ludwig, 1967; McGinley, *et al.*, 1980). We hypothesize that occurrence of porphyrins *in vivo* could be due to their biosynthesis by propionibacteria in response to raised oxygen tension within the pilo sebaceous duct. Release of porphyrins by this flora would accelerate squalene oxidation, and thus rapidly restore the micro-aerophilic conditions required by the bacteria. Further studies are needed to assess a possible influence of oxygen tension on porphyrin biosynthesis by propionibacteria.

In the present study, squalene has been shown to be a highy effective oxygen-trapping compound. This agrees with previous findings where a positive correlation was found between quantities of sebum and colonization by propionibacteria, on different skin sites (McGinley *et al.*, 1980). Such a close link in the light of the findings reported here suggests the need for re-appraisal of the role of sebum, a long debated question (Kligman, 1963), as squalene would appear to be the factor which may control the colonization of the skin by micro-aerophilic flora.

This study has confirmed that oxidized squalene has comedogenic properties, which is in agreement with previous results (Mills, Porte & Kligman, 1978; Motoyoshi, 1983).

Regarding squalene derivatives, further chemical analysis (including mass spectrometry) is needed to elucidate the mode of action of compound D, in the process of comedogenesis. In addition, the relationship between comedo formation and subsequent bacterial colonization needs to be confirmed, in an *in vivo* situation, to assess the physiological role of squalene oxides in the pathogenesis of acne.

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# A possible role for squalene in the pathogenesis of acne. II. *In vivo* study of squalene oxides in skin surface and intra-comedonal lipids of acne patients

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## SUMMARY

Skin surface lipids and lipids from open and closed comedones in acne patients were analysed by thin layer chromatography. The results showed that these lipids were enriched in polar lipids, as compared with the skin surface lipids obtained from controls without acne. In both open and closed comedones, these polar lipids appeared to be derived mainly from the oxidation of squalene, which is in agreement with our previous *in vitro* results.

We suggest that squalene oxidation is the link between comedogenesis and bacterial colonization, and based on this, we propose a hypothesis of the pathogenesis of acne.

'Sebum fuels the acne flame' (Plewig & Kligman, 1975) is a dogma which clearly symbolizes the close dependency of acne on the sebaceous gland. The most recent evidence of the relevance of this dogma comes from studies dealing with the effect of 13 *cis*-retinoic acid. When given orally in man, this compound reduces the sebaceous glands, in few months, to a fine epithelial cord (Jones, Blanc & Cunliffe, 1980), resulting in a sharp decline in the sebum excretion rate (SER), (Strauss, Stranieri & Farrell, 1980; Leyden & McGinley, 1982; Jones, Cunliffe & Cove, 1981), associated with a rapid decrease in the number of inflamed and non-inflamed acne lesions.

From a quantitative viewpoint, acne patients have been clearly characterized as high sebum producers, and severity of acne is positively correlated with SER (Cunliffe & Shuster, 1969). However, from an analytical point of view, the distinction between sebaceous lipids of acne patients, compared with those of normal subjects, has been conflicting and therefore misleading (*see* review by Cunliffe & Cotterill, 1975).

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Most of the studies dealing with qualitative analysis of sebaceous lipids, extracted from sebaceous-rich regions such as the forehead, have been carried out by thin layer chromatography (TLC). This allows the separation of the main classes which compose the skin surface lipids (Downing, 1968; Ruggieri *et al.*, 1982; Nicolaides *et al.*, 1970; Greene *et al.*, 1970). After migration, the various lipidic components are charred, so that they appear as black spots, and their quantities can be determined by photodensitometric measurements. In this way, the SSL have been found to have the following composition, in increasing order of polarity: squalene, sterol-esters, wax-esters, triglycerides, free fatty acids, cholesterol and mono- and diglycerides. However, too little attention has been paid to the black spot on the TLC plates located where the lipid mixture was applied before migration. In the classical 3 bath system (Downing, 1968), this spot does not migrate, and therefore has been neglected.

The aim of this part of the study, taking into account previous studies (Motoyoshi, 1983; Fournier, Emerit & Michaelson, 1980; Ohkido, Yoshino & Matsuo, 1979; Saint Leger *et al.*, 1986) was to assess to what extent the compound producing this spot, initially described by Nicolaides *et al.* (1970), is a sebaceous product and furthermore, to determine if this compound plays a role in the pathogenesis of acne following our earlier finding (Saint-Leger *et al.*, 1986) that squalene oxides exhibit the same chromatographic behaviour.

## METHODS

### *Patients*

Thirty acne patients, 19 males and 11 females aged 14–39 years, were examined at the Hôpital Saint Louis, Paris. The patients had mild to severe nodulo-cystic acne, and none was receiving any treatment.

### *Sampling of skin surface lipids*

The forehead of each patient was cleaned by wiping three times with cotton-wool soaked in a 70% ethanol solution. Thirty and 120 min later, surface lipids were sampled by a ground glass plate technique, using 6 mm diameter platelets, as previously described (Saint-Leger & Bague, 1981). The platelets were pressed, under constant pressure, onto different micro-regions of the skin, i.e. acne lesions (open comedones, closed comedones, papules, nodules, etc.) and on uninvolved skin, in a procedure similar to that used in previous studies (Nicolaides *et al.*, 1970); Simpson, 1983). As a control, samples were also obtained from 18 volunteers with a healthy skin, who were in the same age range and had comparable SERs (1·2–2·3 $\mu g/cm^2/min$).

### *Comedone extraction*

The lesions from which surface lipids were collected were subsequently extracted using a comedo extractor in the case of open comedones. Closed comedones were initially incised using a sterile lancet, allowing their extrusion. The samples were then individually placed into microtest tubes (Waters-HPLC equipment) prior to analysis.

### *Lipid analysis*

*Surface lipids.* The glass platelets carrying the surface lipid samples were applied to the pre-absorbent area of TLC plates (Whatman–LHP-K) according to a technique previously described (Saint-Leger & Bague, 1981). At each edge of the TLC plate, 5 $\mu l$ of an artificial mixture of lipids were deposited as external standards. The migration process was carried out using the four successive solvent systems reported earlier (Saint-Leger *et al.*, 1986).

*Comedonal lipids.* One ml of a 2:1 chloroform:methanol mixture was poured into each microtest tube. Material from each acne lesion (65 open comedones and 33 closed comedones) was rapidly disrupted by means of mechanical action, using small glass capillaries, and sonicated for 1 h. The solution was filtered (Millipore, solvent filtration kit), evaporated under vacuum, and the residue was kept under nitrogen at −20°C prior to analysis, which was performed as follows: 100 $\mu$l of 2:1 chloroform:methanol solution were added to each tube, and aliquots of 5, 10 and 20 $\mu$l of each sample were deposited, alongside external standards, onto the pre-absorbent area of TLC plates. These were treated as above.

### *Bacteriological and lipid profiles of comedones*

This experiment was carried out on 25 open comedones and 21 closed comedones in an attempt to correlate, in the same sample, the concentration of a particular class of lipid with the density of bacterial colonisation, mainly *Propionibacterium acnes.*

### *Bacteriological profile*

After extraction, each lesion was placed into a sterile microtest tube (Waters), and 1 ml of sterile saline was added. After mechanical disruption using sterile glass capillaries, 0·5 ml of the saline suspension was taken off and a 10-fold dilution series was prepared in Triton (0·1%) phosphate buffer (0·075 M, pH 7·9) (Williamson & Kligman, 1965). 100 $\mu$l of each dilution was plated onto a selective medium for propionibacteria (Reinforced Clostridium Medium, Oxoid CM 151) with 6 $\mu$g/ml of furazolidone. The plates were incubated for seven days at 36°C in anaerobic jars, using the Gaspak system (BBL). After incubation, the number of colonies, was recorded, and the density of propionibacteria in each lesion determined.

### *Lipid profiles*

Chloroform, 2 ml (analar grade) was then added to each microtest tube and the two phases were vortexed and then sonicated for 1 h. The mixture was then transferred to micro decantation flasks and chloroform phase was decanted, filtered, washed twice with distilled water, evaporated under vacuum, and analysed as above. This lipid extraction method, when applied to known amounts of an artificial lipid mixture, resulted in very little loss of lipid material ($<2\%$) relative to physical extraction of lipids with saline, and did not cause artefactual oxidization of the squalene included in the mixture.

### *Calculations*

After charring, the amounts of the different lipid classes in each sample were measured by photodensitometry (Shimadzu CS 920), using the external standards for calibration.

The amount of the most polar lipid, referred to as compound D, present in each sample was determined by *initially assuming* a complete identity with compound D obtained by *in vitro* photo-oxidation of squalene, taking into account the constant ratio of 0·52 between the photodensitometric readings for compound D and those for pure squalene included in the external standards. For each sample, the relative composition of the surface lipids or intra-comedonal lipids was determined according to the formula:

$$X\% = \frac{MX}{\Sigma M} \times 100$$

where $MX$ is the amount of compound $X$ and $\Sigma M$ is the sum of the amounts of the following classes of lipids: squalene, wax esters and sterol esters, triglycerides, free fatty acids, cholesterol and compound D.

## RESULTS

### *Analysis of skin surface lipids*

Table I shows that SSL from the acne patients tended to have a lower squalene concentration compared with SSL from the control subjects, and this appeared to be balanced by an increase in compound D. No statistically significant differences between the two groups were found for the other lipid classes (wax esters, triglycerides and free fatty acids). Attempts to establish a specific lipid profile for each type of lesion (open and closed comedones, papules) and uninvolved skin, failed.

In acne patients, compounds were detected which had the same $R_f$ values as those of the squalene oxidation products A, B, C, and D described previously (Saint-Leger *et al.*, 1986) while in control subjects, trace amounts of compound D only were found, although the total amounts of lipids collected were comparable (15–60 μg). The photodensitometric analysis of the cholesterol spot may in fact be quantifying both cholesterol and compound C. The increase in 'cholesterol + compound C' in acne patients (Table I) was probably an increase in the concentration of compound C rather than pure cholesterol, in view of the presence of the squalene oxidation products A, B, and D.

### *Comedonal lipids*

The pattern of lipids in open and closed comedones (Figs 1 and 2) appeared to be different from that of surface lipids, showing a large increase in compound D, and a parallel decrease in the content of pure squalene (Table 2). In closed comedones there was a significantly larger quantity of compound D as compared with open comedones ($P < 0{\cdot}005$). Neither compound A nor compound B was visible in the lipid profiles from either type of lesion.

TABLE 1. Concentration of squalene and its derivatives in the SSL of acne patients and normal subjects

| Lipid class | Percentage of SSL (mean ± SD) | |
|---|---|---|
| | Acne patients | Control subjects |
| Squalene | 10·7 ± 4·5 | 12·3 ± 2·9 |
| Compound A | 4·3 ± 2·7 | Traces |
| Compound B | 2·8 ± 2·0 | Traces |
| Cholesterol and compound C* | 2·2 ± 0·7 | 1·7 ± 0·6 |
| Compound D | 0·7 ± 0·3 | Traces |

* Compound C was found to have the same $R_f$ value as cholesterol.

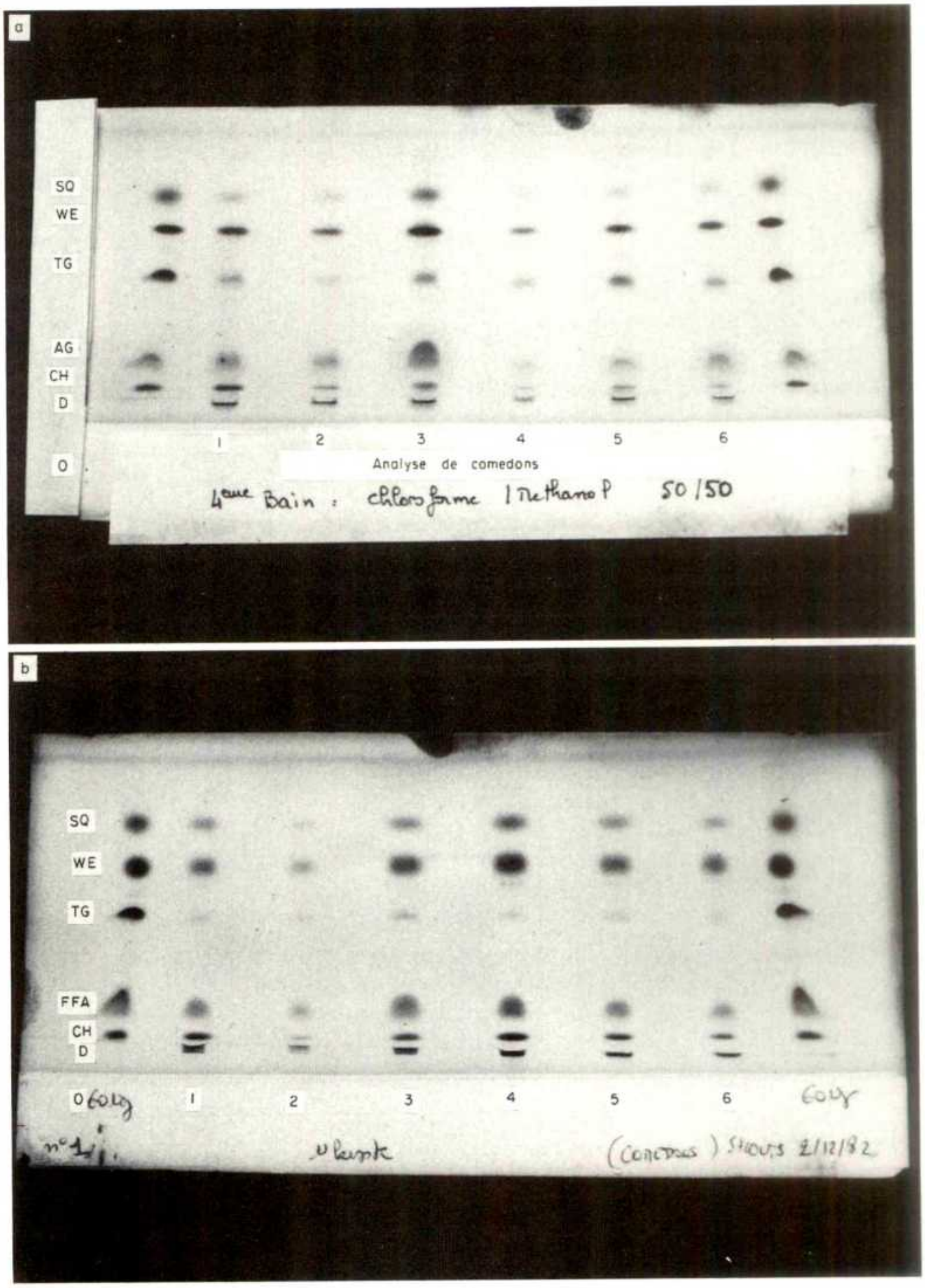

FIGURE 1. Illustration of the chromatographic pattern of lipids extracted from individual open comedones (a) and closed comedones (b). The two outer lanes contain standards. O = origin; SQ = squalene; WE = wax esters; TG = triglycerides; FFA = free fatty acids; CH = cholesterol.

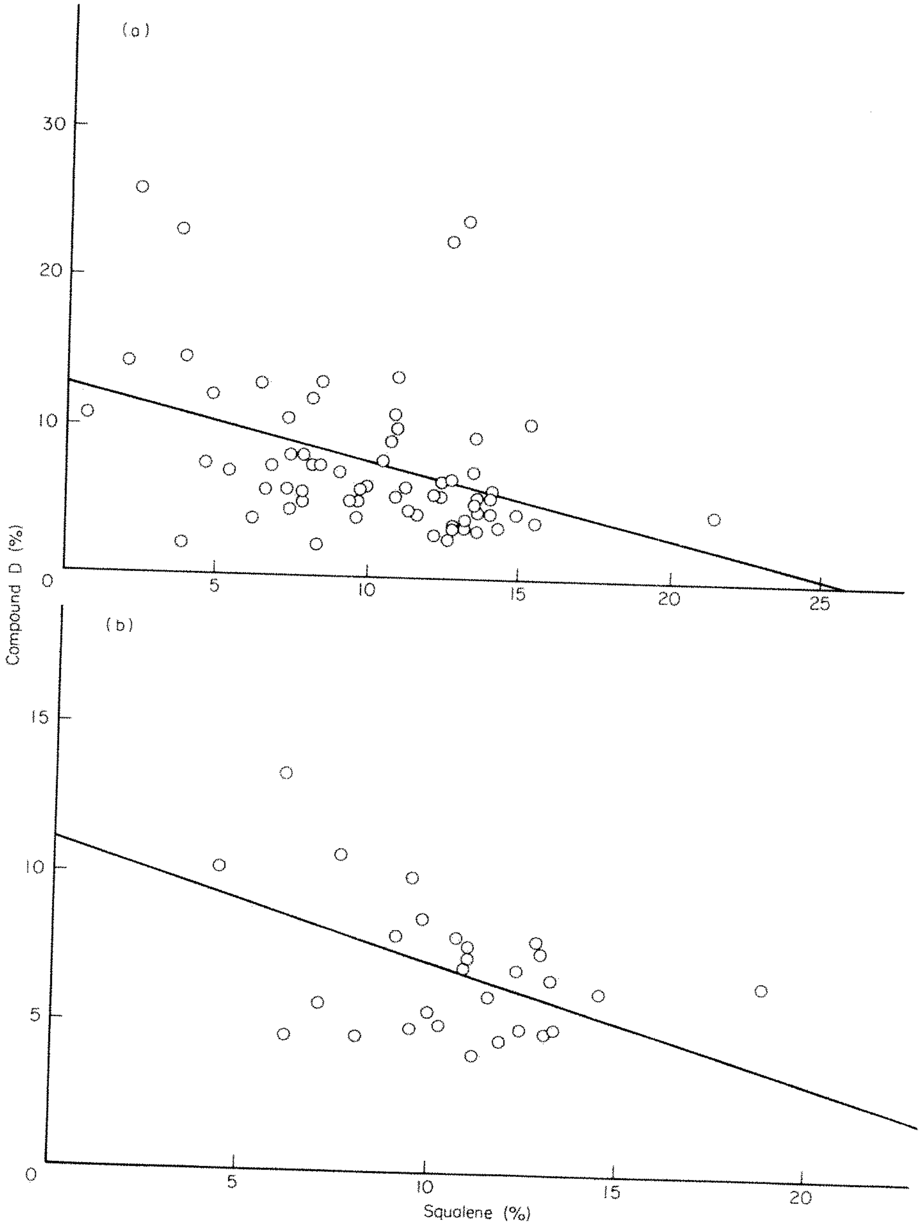

FIGURE 2. Relationship between the content of squalene and compound D in (a) open comedones $n = 65$; $r = 0{\cdot}37$; $P < 0{\cdot}005$, and (b) closed comedones $n = 33$; $r = 0{\cdot}46$, $P < 0{\cdot}01$.

There was a significant inverse correlation between the concentrations of compound D and squalene in both types of lesions (Fig. 2a, b). Such a correlation was not observed in the skin surface lipids of acne patients, which suggests that conversion of squalene to compound D is a characteristic of the infundibulum of the pilosebaceous ducts.

No significant correlation was found between the concentration of compound D and the

TABLE 2. Concentration of squalene and its derivatives in open and closed comedones

| Lipid class | Percentage of comedonal lipids (mean ± SD) | | *P* (Student's *t*-test) |
|---|---|---|---|
| | Open comedones | Closed comedones | |
| Squalene | 10·1 ± 3·8 | 10·8 ± 3·6 | NS |
| Compound A | ND | ND | — |
| Compound B | ND | ND | — |
| Cholesterol + compound C* | 4·8 ± 1·9 | 4·4 ± 2·4 | NS |
| Compound D | 7·7 ± 5·1 | 10·7 ± 2·9 | <0·005 |

* Compound C has the same $R_f$ value as cholesterol.
ND = not detected.

density of colonization by propionibacteria (Fig. 3). This suggests that the presence of oxidized squalene does not affect the proliferation of these bacteria, once a suitable micro-aerophilic environment has been established.

## DISCUSSION

From the results reported here, two major findings have emerged. Firstly, in acne patients, surface and comedonal lipids were found to differ from the surface lipid pattern found in normal subjects in that there was a higher content of the most polar lipids, shown mostly to originate from squalene. In some patients, compound D was found to comprise up to 22% of the comedonal lipids without, however, any apparent correlation with the severity of the acne in these patients.

The question of the extent to which such a high concentration of oxidized material may affect the development of the lesion requires further study. However, in comedonal lipids, the intermediate products, compounds A and B, were not detected, suggesting that the terminal oxidation product of squalene oxidation, compound D, could be causally related to comedo formation. The inverse correlation between the concentrations of squalene and compound D, obtained in our study, is in good agreement with the findings of Nicolaides *et al.* (1970). This suggests that there is interdependence between these compounds *in vivo* and that compound D originates largely from squalene, although it is possible that other oxidized lipids (oxidized fatty acids, oxidized cholesterol etc.) may exhibit the same chromatographic behaviour.

Secondly, squalene oxidation appears, in the pathogenesis of acne, as a promoting factor in comedogenesis and, as a secondary effect may encourage the proliferation of micro-aerophilic flora due to the large amount of atmospheric oxygen bound during the oxidative process (Saint-Leger *et al.*, 1986). The proposed order of events is illustrated in Figure 4 and is consistent with the findings of Lavker, Leyden & McGinley (1981) who presented evidence that the early formation of comedones does not require the presence of any bacteria. Our *in vitro* results showed that squalene oxidation could occur without the presence of a catalyst. It is tempting, therefore, to equate compound D with the lipid droplets found by Lavker *et al.* (1981) on

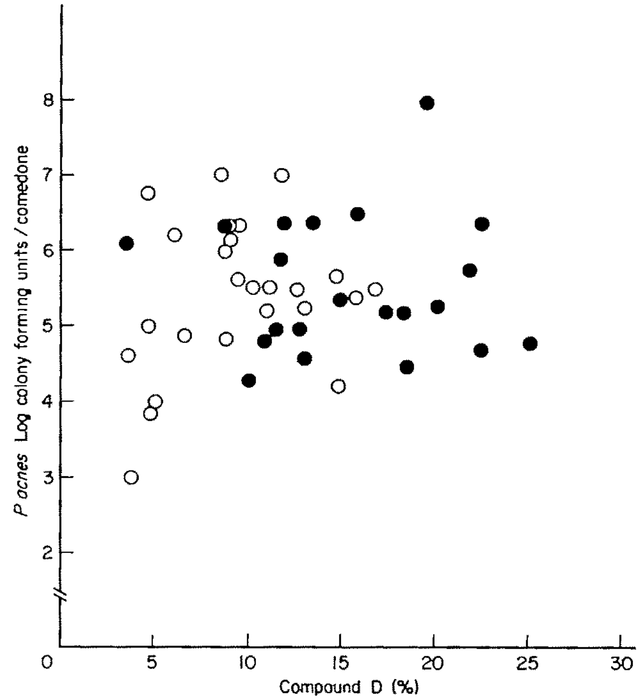

FIGURE 3. Relationship between colonization with *P. acnes* and concentration of compound D, in 25 open comedones (O) and 21 closed comedones (●).

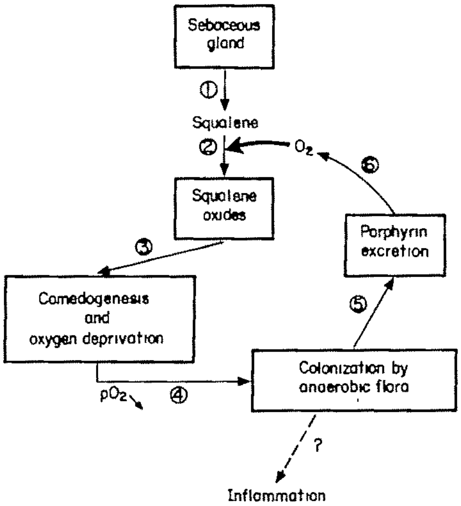

FIGURE 4. Summary of our hypothesis of the pathogenesis of acne, illustrating the proposed succession of events leading to comedogenesis and bacterial colonization. The mechanism by which the inflammatory reaction proceeds is not included.

electron microscopic examination of follicular casts in prepubertal children, which filled the stratum corneum cells and gave them a 'balloon' shape.

It is necessary to determine the degree of squalene oxidization in single normal follicles, in comparison with single comedones. In its early stages, acne involves a few single follicles and much is to be learned about the way by which these specifically undergo comedo formation. It is also important to discover why these events do not occur in normal individuals. Steps 2 and 3, in Figure 4, may differentiate the normal population from acne patients. The presence of membrane-protecting factors, hydrolyzing enzymes or other factors under genetic control may explain why the normal population never develop acne.

The hypothesis proposed in Figure 4 does not explain the way in which an inflammatory reaction, which is probably dependent on bacterial infection, is produced, although squalene oxides may have intrinsic cytotoxic and inflammatory properties (Fournier *et al.*, 1980; Imaeda, Ohsawa & Yokota, 1983). Moreover, closed comedones referred to as 'time-bombs' in the inflammatory process (Plewig & Kligman, 1975), contain a higher concentration of compound D, as compared with open comedones. This hypothesis also does not explain why and how summertime tends to improve acne despite increased UV and visible radiation which may generate singlet oxygen, nor does it explain how acne vulgaris spontaneously resolves. Formation of squalene oxides which can be potentiated by the catalytic action of porphyrin, may play an important role in the initiation of chloracne, as it has been shown that the chlorinated compounds exert a strong porphyrinogenic action (Mensink & Strik, 1982).

The present study suggests that squalene, as a close link between comedogenesis and bacterial colonization, is strongly implicated in the early development of acne.

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# Arachidonic acid metabolism by peripheral blood cells in psoriasis

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## SUMMARY

The metabolism of arachidonic acid by mixed suspensions of leukocytes and platelets prepared from peripheral blood has been studied in 20 patients with psoriasis and 21 healthy controls. A lipoxygenase-derived product, identified as 12,20-dihydroxy-5,8,10,14-eicosatetraenoic acid was formed in increased amounts by the cell suspensions from the psoriatic patients. This product results from the metabolism of platelet-derived 12-hydroxy-5,8,10,14-eicosatetraenoic acid by the polymorphonuclear leukocyte 20-hydroxylase enzyme. By contrast, synthesis of the cyclo-oxygenase products 12-hydroxy-5,8,10-heptadecatrienoic acid and thromboxane $B_2$ was diminished. Benoxaprofen, which is known to be beneficial in psoriasis, diminished the levels of 12,20-dihydroxy-5,8,10,14-eicosatetraenoic acid formed *in vitro*.

A number of studies have suggested an imbalance of arachidonic acid metabolism in psoriasis. Penneys *et al.* (1975) identified an inhibitor of prostaglandin synthesis in psoriatic plaque, and subsequently raised levels of arachidonic acid and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) have been demonstrated in lesional psoriatic skin, together with smaller elevations in the levels of prostaglandin $E_2$ ($PGE_2$) and prostaglandin $F_{2\alpha}$ (Hammarstrom *et al.*, 1975). More recently, increased concentrations of leukotriene $B_4$ ($LTB_4$) have been demonstrated in psoriatic skin (Brain *et al.*, 1982; Grabbe *et al.*, 1984), and the presence of increased levels of arachidonic acid and 12-HETE have been confirmed (Barr *et al.*, 1984a).

It is not yet clear whether the abnormalities that are evident in psoriasis are fundamental to the disease process or are a consequence of it. Forster *et al.* (1983) demonstrated increased activity of phospholipase $A_2$ (an enzyme that releases arachidonic acid from phospholipids) in non-lesional psoriatic skin. This finding suggests that abnormal arachidonate metabolism may be fundamental to the disease. However, in other studies (Brain *et al.*, 1982; Barr *et al.*, 1984a), significant elevations of arachidonate metabolites have been observed only in lesional epidermis.

An oral presentation based on this work was presented at the Annual Meeting of the British Society for Investigative Dermatology 1984 (Abstract: *British Journal of Dermatology*, 1984, **111**, 706).

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In addition, elevated levels of cutaneous $LTB_4$ have been shown in other inflammatory dermatoses, such as contact dermatitis and atopic eczema (Barr *et al.*, 1984b; Ruzicka *et al.*, 1984). These findings suggest that the abnormal arachidonate metabolism in psoriasis may be secondary to cutaneous inflammation.

There are several agents that have been used in the treatment of psoriasis that are now known to inhibit the lipoxygenase enzymes. These include anthralin (Bedord, Young & Wagner, 1983), etretinate (Sircar & Schwender, 1983) and benoxaprofen (Walker & Dawson, 1979; Allen & Littlewood, 1982; Harvey *et al.*, 1983; Kragballe & Herlin, 1983); although there is some doubt about the 5-lipoxygenase inhibitory activity of the latter (Masters & McMillan, 1984; Salmon *et al.*, 1984). By contrast, the cyclo-oxygenase inhibitors indomethacin and phenylbutazone can exacerbate psoriasis (Katayama & Kawada, 1981; Ellis *et al.*, 1983; Reshad, Hargreaves & Vickers, 1983). These observations might also suggest an imbalance of arachidonate metabolism in psoriasis.

Studies of polymorphonuclear leukocyte (PMNL) chemotaxis and chemokinesis (Wahba *et al.*, 1978; Kawohl *et al.*, 1980; Breathnach, Carrington & Black, 1981; Preissner, Schroder & Christophers, 1983; Fraki *et al.*, 1983), adherence (Sedgwick, Bergstresser & Hurd, 1980), phagocytosis (Wahba *et al.*, 1978), superoxide generation and degranulation (Preissner *et al.*, 1983; Sedgwick, Bergstresser & Hurd, 1981) and enzyme activities (Glinski *et al.*, 1984) have been performed in psoriasis, mostly, but not always, with abnormal results. These abnormalities have often appeared to be secondary to active disease. Since lipoxygenase metabolites of arachidonic acid, especially $LTB_4$, are important mediators of some of these functions in the PMNL (Ford-Hutchinson *et al.*, 1980; Goetzl, Woods & Gorman, 1977; Bokoch & Reed, 1981), it is important to know whether the metabolism of arachidonic acid in the peripheral blood cells is normal.

In a recent study (Maurice, Bather & Allen, 1986), no abnormality of arachidonate metabolism was found in purified suspensions of peripheral blood PMNL in psoriasis. However, it is possible that interaction between the PMNL and mononuclear white blood cells or platelets is important, and in the present study we have, therefore, investigated arachidonate metabolism in a mixed population of peripheral blood cells from psoriatic patients and normal controls.

## METHODS

### *Patients*

Twenty patients with chronic plaque psoriasis (9 male, 11 female, mean age 33 years) and 21 normal controls (11 male, 10 female, mean age 38 years) were included in the study. The patients selected had no medical problems other than psoriasis, and the controls were healthy individuals who had no first-degree relatives with psoriasis. None of the patients had received any systemic anti-psoriatic treatment for at least 3 months prior to venepuncture, and were asked to discontinue all topical therapy except white soft paraffin for 3 days. None of the patients or controls was on any medication, including the contraceptive pill, and all denied taking any preparation containing aspirin during the preceding 10 days. The extent of the psoriasis was noted in each case and ranged from under 5% of the body surface area (a few localized plaques) to 65% (sub-erythrodermic).

### *Materials*

[1-$^{14}$C]-arachidonic acid ($^{14}$C-AA) (specific activity 58·4 mCi/mmol) was purchased from the

Radiochemical Centre, Amersham, U.K. After removing the toluene solvent under nitrogen, the free acid was resuspended in a small volume of ethanol, then diluted in Dulbecco's buffer (see below); the amount of ethanol used was such that its concentration never exceeded 0·25% in the cell incubations. Indomethacin, a cyclo-oxygenase inhibitor, was purchased from the Sigma Chemical Co. BW755C, a cyclo-oxygenase and lipoxygenase inhibitor (Salari, Braquet & Borgeat, 1984) was a gift from the Wellcome Research Laboratories, Beckenham, Kent, U.K. Nordihydroguaiaretic acid (NDGA), predominantly a 5-lipoxygenase inhibitor, but also an inhibitor of 12-lipoxygenase and cyclo-oxygenase at higher concentrations (Salari *et al.*, 1984) was purchased from the Sigma Chemical Co. A23187, a calcium ionophore, which stimulates the release of arachidonic acid (AA) from cell membrane phospholipid and its subsequent metabolism by lipoxygenase (Borgeat & Samuelsson, 1979), was also obtained from the Sigma Chemical Co. Benoxaprofen was a gift from Lilly Research Centre, Windlesham, Surrey, U.K. All incubations were carried out in Dulbecco's buffer (phosphate-buffered saline containing 0·5 mM $MgCl_2$ and 0·9 mM $CaCl_2$, pH 7·3). All the standards were a gift from Dr J. Walker, Lilly Research Centre, except $PGE_2$ (Sigma Chemical Co.), thromboxane $B_2$ ($TXB_2$) (Radiochemical Centre, Amersham, U.K.) and 12,20-dihydroxy-5,8,10,14-eicosatetraenoic acid (12,20-diHETE) which was a gift from Dr J. Rokach, Merck Frosst Laboratories, Quebec, Canada.

### *Blood collection*

Venous blood was taken under standardized conditions and collected into tubes that contained di-sodium EDTA (final concentration 4 mM). 5 ml of the anticoagulated blood was used for a platelet count and 20 ml used to prepare the mixed cell suspension. In some experiments, a further 20 ml was used to prepare a suspension of washed platelets.

### *Preparation of mixed cell suspensions*

The red cells were sedimented by adding 4 ml of a solution of 6% (w/v) dextran (M.W. 151 000) in saline and allowing the mixture to stand undisturbed for 20 min, after which the supernatant was centrifuged at 160 ***g*** for 10 min. Any red cells that remained in the pellet thus obtained were lysed by resuspending the cells in 0·2% (w/v) saline for 1 min, after which isotonicity was restored by adding 1·6% (w/v) saline. After further centrifugation, the cells were washed twice in Dulbecco's buffer and finally resuspended in the same medium. The volume of medium used was such that the mixed cell preparation always contained $1{\cdot}5 \times 10^7$ white cells/ml. A manual platelet count and a differential white cell count were performed on each sample. The final suspension, therefore, contained a constant number of white cells, consisting of a mixture of PMNL and mononuclear leukocytes (MNL). In all the 41 mixed-cell preparations the ratio of PMNL to MNL was approximately 4:1. The numbers of platelets varied between $2 \times 10^6$ and $7 \times 10^7$/ml.

### *Preparation of suspensions of washed platelets*

Anticoagulated blood was centrifuged at 160 ***g*** for 10 min and the upper turbid layer of platelet-rich plasma removed. This was further centrifuged at 2300 ***g*** for 10 min and the platelet pellet washed twice using 0·02 M Tris-HCl buffer, pH 7·6, containing 2 mM di-sodium EDTA. The platelets were finally resuspended in calcium- and magnesium-free phosphate-buffered saline (pH 7·3).

### *Incubation of cell suspensions with $^{14}$C-arachidonic acid*

Mixed cell suspensions from all 41 patients and controls were incubated with $^{14}$C-AA and then analysed. In some experiments the number of platelets in a mixed-cell suspension was increased by the addition of autologous platelet suspension, while maintaining the white cell count constant at $1{\cdot}5 \times 10^7$/ml.

Incubations were carried out in duplicate. 0·5 ml samples of the cell suspension were stirred at 37°C for 5 min, and 100 μl of the stock solution of $^{14}$C-AA (0·1 μCi) was then added. The final concentration of AA in the incubations was always 3·4 μM. After a further 5 min, the reaction was terminated by acidifying the sample to pH 3 with 20 μl of 0·25 M HCl and diluting with 0·75 ml of ice-cold water.

### *Effect of pharmacological inhibitors, calcium ionophore and benoxaprofen*

In some experiments various pharmacological inhibitors were added to the suspensions 5 min prior to the $^{14}$C-AA. These were indomethacin (final concentration $10^{-4}$M), BW755C ($1{\cdot}6 \times 10^{-5}$ M) and NDGA ($5{\cdot}3 \times 10^{-6}$ M). Benoxaprofen ($1{\cdot}7 \times 10^{-5}$ M) was also used. In order to study the effect of calcium ionophore, A23187 (final concentration $3{\cdot}8 \times 10^{-6}$M) was added to the incubation at the same time as the $^{14}$C-AA.

### *Extraction of samples and separation of products*

Samples were extracted twice with ethyl acetate (2 ml), dried under nitrogen, redissolved in a small volume of ethyl acetate, and applied to one lane of a multi-lane thin-layer chromatography (TLC) plate (Whatman LK6D). The plates were developed in toluene: dioxane: glacial acetic acid (65:34:1·5 by volume) at 4°C (Harvey & Osborne, 1983). In this system, a good separation of cyclo-oxygenase and lipoxygenase products was achieved. The plates were then scanned, using a Panax Thin Layer Scanner RTLS-1A, with argon quenching, to locate the peaks of radioactivity. The silica gel was scraped from these areas and counted in a liquid scintillation counter. The radioactivity (cpm) in each peak was expressed as a percentage of the total recovered radioactivity from each lane.

### *Identification of products derived from $^{14}$C-arachidonic acid*

The major products were identified by comparing their $R_f$ values with those of authenticated standards applied to the same plate. The standards and their $R_f$ values in the solvent system used were $PGE_2$($R_f$ 0·25), $TXB_2$ ($R_f$ 0·31), $LTB_4$ ($R_f$ 0·53), 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE, $R_f$ 0·65), 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT, $R_f$ 0·68), and 12-HETE ($R_f$, 0·72).

### *Confirmation of product identification using high-performance liquid chromatography*

To confirm the identity of the products formed during the incubation of mixed cells with arachidonic acid, cell suspensions were prepared from 120 ml of blood, incubated and extracted as described, and the extracts pooled. The ethyl acetate was evaporated under nitrogen and the residue resuspended in 600 μl of methanol:water (65:35), ready for analysis by reverse phase HPLC (RP-HPLC). This was done using the Hewlett-Packard 1084B system with a diode array UV detector, and a 150 mm × 3·9 mm resolve $C_{18}$ column, 5 μM particle size (Waters, Harrow, Middlesex, U.K.). The mobile phase was water (containing 0·05 M ammonium acetate) and methanol, pH 7·0, using a gradient elution. The methanol was increased from 55% to 65% linearly over 10 min, with a flow rate of 2 ml/min. The temperature was set at 35°C. In this system, the retention times of standards were as follows: HHT 4·6 min, $LTB_4$ 5·5 min, 12-HETE 11·4 min. The solvent fractions eluting off the column which showed peaks of UV absorption were collected for scintillation counting.

### Product identification by gas chromatography-mass spectrometry

The product for identification was analysed by gas chromatography-mass spectrometry according to the method of Mallet, Barr and Newton (1986).

### Statistical analysis

Non-parametric methods were used: the Mann-Whitney test and Spearman's rank correlation (MINITAB Statistical Software Package: Copyright Pennsylvania State University). A significance level of 0·05 was used in all cases.

## RESULTS

### Products formed after incubating mixed cell suspensions with $^{14}$C-arachidonic acid

Incubation of mixed-cell suspensions from psoriatics and controls with $^{14}$C-AA yielded radioactive compounds which co-migrated with both cyclo-oxygenase- and lipoxygenase-derived products. The cyclo-oxygenase-derived products were identified as $TXB_2$ and HHT, and the lipoxygenase-derived product as 12-HETE. In addition, there was a major radio-labelled compound ($R_f$ 0·57) in samples from all 41 subjects, which chromatographed close to the $LTB_4$ standard ($R_f$ 0·53). This is thought to be 12,20-diHETE and the evidence for this is given below. No $LTB_4$ or 5-HETE were present in any of the samples. Some polar lipids (material remaining near the origin) and neutral lipids (material migrating with the solvent front) also became radio-labelled. A typical radiochromatogram is shown in Figure 1.

### Levels of arachidonic acid metabolites

Levels of arachidonic acid metabolites formed by mixed-cell suspensions from psoriatic patients and controls are shown in Figure 2. Formation of 12,20-diHETE by mixed cells was significantly elevated in the psoriatic group, with a median value of 15·7% compared with 12·7% in the controls ($P = 0·02$). HHT formation, by contrast, was significantly lower in the psoriatic group, with a median value of 5·1% compared with 6·9% in the controls ($P = 0·02$). Production of $TXB_2$ was also lower in the psoriatic group (7·3%) compared with the controls (8·3%), but this difference was not statistically significant. There was no significant difference between controls and psoriatics in the production of 12-HETE (12·4% and 11·2% respectively), nor in the amount of AA remaining unconverted after 5 min (6·7% and 7·9% respectively).

There was no significant difference between the differential white blood cell counts in the mixed cell suspensions from controls and psoriatics (median neutrophil counts 71% and 75% of the total white cell count respectively).

The platelet count varied between the different mixed-cell preparations and this may have influenced the levels of metabolites observed. The platelet counts in the mixed-cell suspensions were significantly higher in the psoriatic group (median $21·5 \times 10^6$/ml; range $8·0–49·0 \times 10^6$/ml) than in the controls (median $13·0 \times 10^6$/ml; range $2·6–23·0 \times 10^6$/ml) ($P = 0·001$). The whole blood platelet counts were also higher in the psoriatics (median $226 \times 10^6$/ml; range 149–$325 \times 10^6$/ml) than in the controls (median $180 \times 10^6$/ml; range $82–247 \times 10^6$/ml) ($P = 0·001$), which could have accounted for the higher platelet count in the mixed-cell suspensions from the psoriatics.

### Effect of the number of platelets in the mixed-cell suspensions on levels of metabolites

There was a significant positive correlation between the $TXB_2$ production and the number of

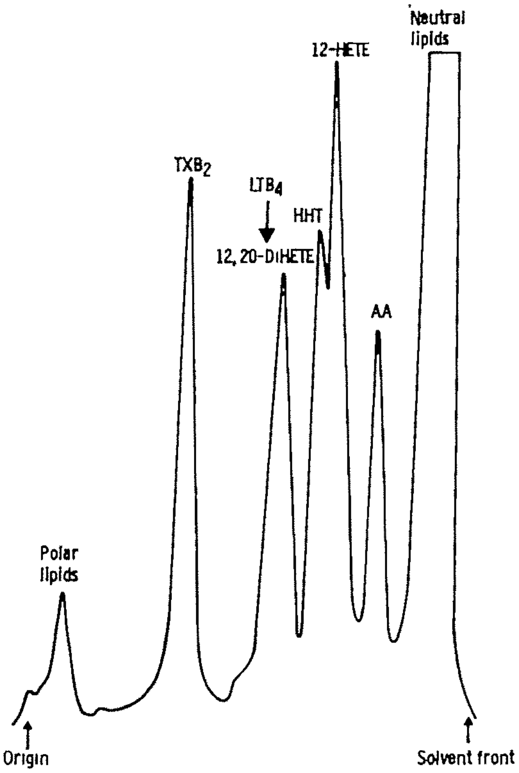

FIGURE 1. A typical thin layer radiochromatogram of the ethyl acetate extract of a mixed peripheral blood cell suspension incubated with $^{14}$C-arachidonic acid, showing the lipoxygenase and cyclo-oxygenase products that are formed. The position of the LTB$_4$ standard is indicated.

platelets in the mixed-cell suspensions ($r=0{\cdot}67$, $P<0{\cdot}001$ in the controls; $r=0{\cdot}48$, $P<0{\cdot}05$ in the psoriatics), the suspensions with higher platelet counts more actively synthesizing TXB$_2$. There was no significant correlation between 12-HETE production and platelet count in the controls or in the psoriatics ($r=0{\cdot}30$ and $0{\cdot}21$, respectively), nor between 12,20-diHETE levels and platelet count in the controls or the psoriatics ($r=0{\cdot}10$ and $0{\cdot}26$, respectively).

In some experiments, the number of platelets in the mixed cell suspension was increased artificially by adding platelets to the preparation (Fig. 3). As expected, the amount of unconverted substrate diminished steadily with increasing platelet count. The amount of 12,20-diHETE remained unaffected over the whole range of platelet counts tested. By contrast, TXB$_2$ increased steadily with platelet count, and this relationship was approximately linear over the range of platelet counts occurring in the mixed-cell suspensions. 12-HETE showed a more complex relationship; at low platelet counts it was formed in excess of TXB$_2$, but production levelled off, and then diminished as the platelet count increased.

Since there was an approximately linear relationship between TXB$_2$ production and the number of platelets in the incubation mixture, the TXB$_2$ production in the mixed-cell suspensions was expressed per $5\times10^5$ platelets (Fig. 4). A significant decrease in TXB$_2$ production by mixed cells from the psoriatics was then apparent, with a median of 0·335% per $5\times10^5$ platelets, compared with 0·640% in the controls ($P=0{\cdot}001$). It was not possible to make

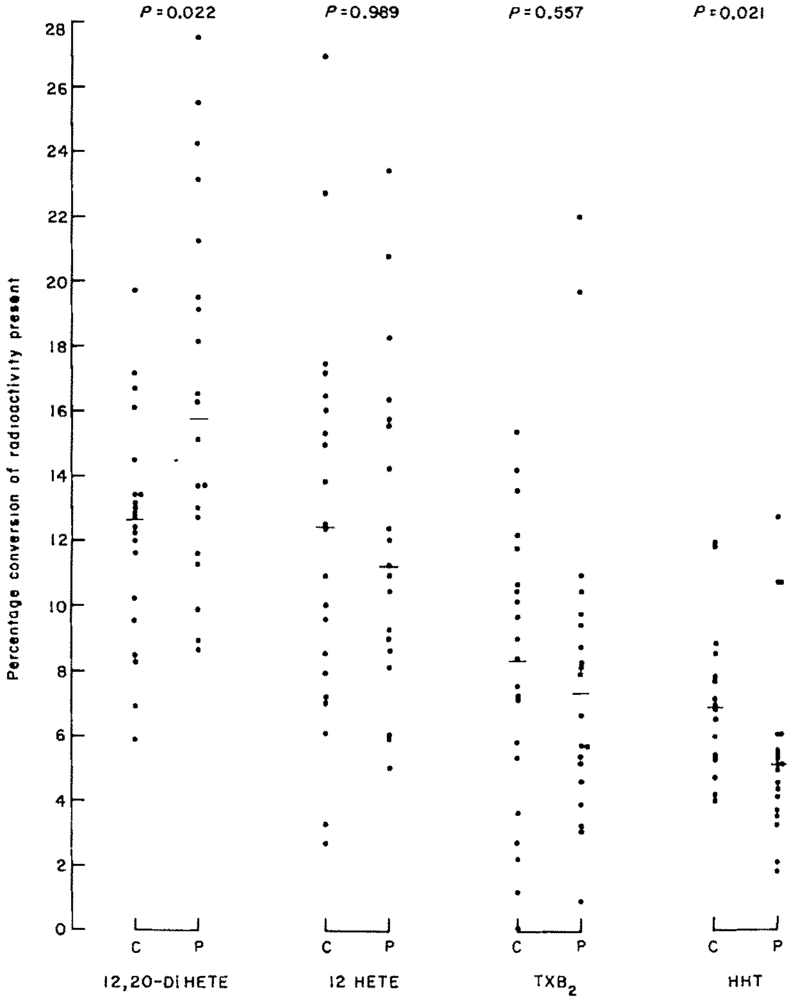

FIGURE 2. Percentage of incorporation of recovered radioactivity into 12,20-diHETE, 12-HETE, $TXB_2$ and HHT, after incubating mixed peripheral blood cells from psoriatic (P) and control (C) subjects with $^{14}$C-arachidonic acid. Bars indicate medians (HHT was measured in cells from only 19 psoriatics and 16 controls).

the same adjustment for platelet count with the 12-HETE values because of the non-linear relationship between 12-HETE levels and platelet count.

### *Effect of extent of psoriasis on levels of metabolites*

There was no significant correlation between the levels of AA metabolites and the percentage of the body surface area affected by psoriasis. This was true for 12,20-diHETE ($r = 0{\cdot}29$) and TXB expressed per $5 \times 10^5$ platelets ($r = -0{\cdot}08$), although the levels of both of these metabolites were abnormal in the psoriatic group as a whole.

### *Effect of benoxaprofen*

The effects of benoxaprofen on production of AA metabolites is shown in Fig. 5. The addition of

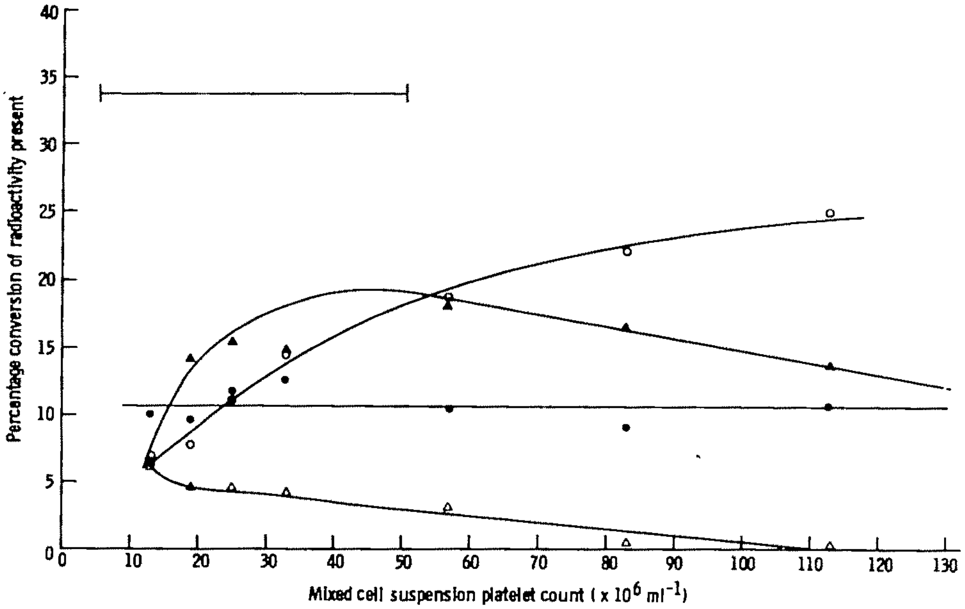

FIGURE 3. The effect of artificially increasing the platelet count in a mixed-cell suspension on the levels of arachidonic acid metabolites (expressed as percentage incorporation of recovered radioactivity). ——— indicates the range for the platelet counts in the mixed cell suspensions. ● 12,20-diHETE; ▲ 12-HETE; ○ $TXB_2$; △ unconverted arachidonic acid.

benoxaprofen to the mixed cell suspensions from eight psoriatic, and one normal, subjects caused consistent inhibition of 12,20-diHETE production. Median inhibition was 35% (range 3%–55%). $TXB_2$ and HHT were also consistently inhibited, by 49% (range 29%–100%) and 48% (range 15%–100%) respectively ($n = 8$). By contrast, 12-HETE was markedly increased, by 78% (range 30%–189%).

### *Effect of pharmacological inhibitors of known specificity and of a calcium ionophore*

Indomethacin abolished $TXB_2$ and HHT production in mixed-cell suspensions from two normal subjects. It had no effect on production of 12,20-diHETE but the amount of radiolabelled 12-HETE was increased by a median of 66% (range 64%–68%). BW755C also abolished production of $TXB_2$ and HHT in mixed-cell suspensions from three normal subjects. It also reduced 12-HETE and 12,20-diHETE production by a median of 100% (ranges 82%–100% and 77%–100% respectively). NDGA abolished $TXB_2$ and HHT production in a mixed-cell suspension from one normal subject; 12-HETE and 12,20-diHETE were reduced by 62% and 68% respectively.

These changes are in accord with the known properties of the various agents and the identities ascribed to the different products. The behaviour of the 12,20-diHETE in these experiments was that expected of a lipoxygenase-derived product.

Addition of the calcium ionophore A23187 to the mixed-cell suspensions from two normal subjects resulted in the abolition of $TXB_2$, HHT, and 12,20-diHETE production. $PGE_2$, $LTB_4$ and 5-HETE were formed instead. 12-HETE was reduced by a median of 53% (range 52%–53%).

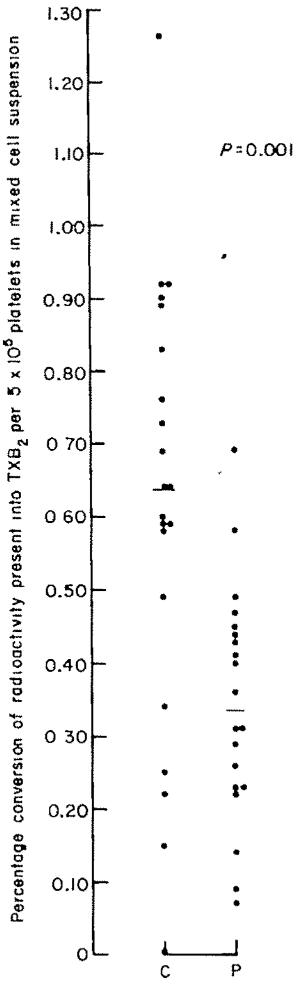

FIGURE 4. Percentage incorporation of recovered radioactivity into $TXB_2$ by mixed cell suspensions from psoriatic (P) and control (C) subjects incubated with $^{14}C$-arachidonic acid. Corrected values expressed per $5 \times 10^5$ platelets..

### *Analysis of products by reverse-phase HPLC*

RP-HPLC of the ethyl acetate extract of mixed cells incubated with $^{14}C$-AA showed three peaks of UV absorbance, at 237 nm, which contained radioactivity. These had retention times of 2·6, 4·6, and 11·4 min respectively. The two compounds that were retained longer were identified as HHT and 12-HETE respectively, by comparison with the retention times of authenticated standards. The peak eluting at 2·6 min, when collected and applied to a TLC plate in the usual solvent system, was shown to have an $R_f$ value of 0·57, and therefore corresponded to the major radioactive peak seen on TLC and was thought to be 12,20-diHETE.

### *Provisional identification of 12,20-diHETE*

This metabolite of arachidonic acid was formed exclusively by the mixed-cell preparations. We

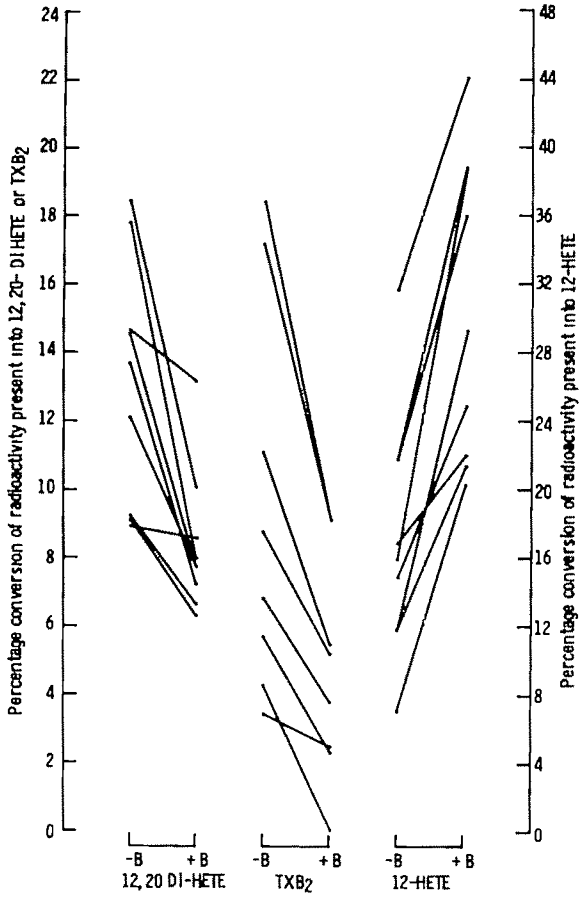

FIGURE 5. The *in vitro* effect of benoxaprofen ($1{\cdot}7 \times 10^{-5}$M) on the levels of 12,20-diHETE, $TXB_2$ and 12-HETE formed (expressed as per cent incorporation of recovered radioactivity) by mixed peripheral blood cells (from eight psoriatic and one control subject) incubated with $^{14}$C-arachidonic acid. −B, without benoxaprofen; +B, with benoxaprofen.

have never seen it when purified platelets or PMNL are incubated alone. Its formation therefore appears to depend upon an interaction between PMNL and platelets and occurs at a concentration of exogenous arachidonic acid (3·4 μM) which activates platelets but is non-stimulatory to PMNL (Marcus *et al.*, 1984). When the PMNL in mixed-cell suspensions are stimulated by A23187, this metabolite is replaced by $LTB_4$ and 5-HETE. The data obtained using pharmacological inhibitors of known specificity indicate that it is a lipoxygenase product.

A UV spectrum of the peak eluting off the HPLC column at 2·6 min is shown in Fig. 6. As can be seen, it has a $\lambda_{max}$ of 236 nm. An authenticated 12,20-diHETE standard was found to have an identical retention time on HPLC to our product.

### *Confirmation by gas chromatography-mass spectrometry*

The identity of the presumed 12,20-diHETE collected off the HPLC column was confirmed by gas chromatography-mass spectrometry.

## DISCUSSION

An increased synthesis of the lipoxygenase-derived product 12,20-diHETE, has been demonstrated in mixed peripheral blood cells of psoriatic patients, incubated with arachidonic acid.

12,20-diHETE is a novel metabolite of arachidonic acid, formed by the action of the $\omega$-hydroxylation system of PMNL on platelet-derived 12-HETE (Wong *et al.*, 1984). Marcus *et al.* (1984) demonstrated that 12,20-diHETE was formed when human platelets were stimulated with thrombin, collagen, or 5·7 $\mu$M arachidonic acid in the presence of PMNL (which remained unstimulated under these conditions). In contrast, when the platelet-PMNL suspensions were exposed to the calcium ionophore A23187, an activator of PMNL 5-lipoxygenase, formation of 12,20-diHETE was suppressed and $LTB_4$ produced instead. Aspirin (a cyclo-oxygenase inhibitor) did not inhibit the synthesis of 12,20-diHETE. The properties described by Marcus *et al.* (1984) for 12,20-diHETE are thus very similar to those of our compound. The $\lambda_{max}$ of 236 nm shown by our compound is what would be expected for 12,20-diHETE (Wong *et al.*, 1984). The identical HPLC retention times of our compound and an authentic 12,20-diHETE standard, add further weight to the identification of the product as 12,20-diHETE, and this was finally confirmed by gas chromatography-mass spectrometry.

Little is known about the biological activity of 12,20-diHETE, but increased production of this compound by mixed cells from psoriatics does indicate an important difference in the metabolic behaviour of the cells. The difference could be due to increased availability of platelet-derived 12-HETE, but this would appear unlikely in view of the fact that quite large changes in the platelet count in the mixed-cell suspensions had no effect on the levels of 12,20-diHETE. Alternatively, the abnormality could be due to an increased activity of the $\omega$-hydroxylation system in psoriatic PMNL.

There was no correlation between the 12,20-diHETE level and the extent of the psoriasis, suggesting that the abnormality may be a primary one, rather than merely a consequence of

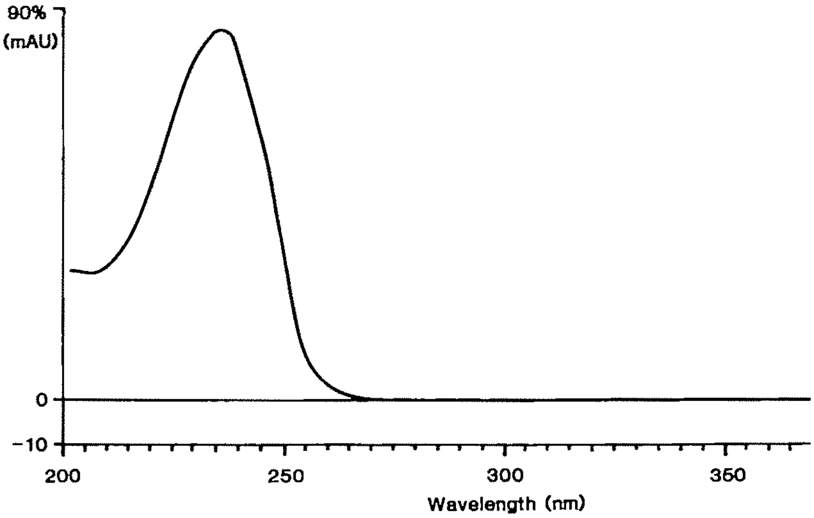

FIGURE 6. UV spectrum of fraction eluting at 2·6 min off reverse-phase HPLC system, during analysis of the ethyl acetate extract of a mixed peripheral blood cell suspension incubated with arachidonic acid, and showing a peak absorbance at 236 nm.

cutaneous inflammation. The considerable overlap, however, between the 12,20-diHETE values in the psoriatic and control groups shows clearly that this factor does not operate alone in the pathogenesis of psoriasis. It would be of interest to observe whether blood cells of psoriatics in remission, or from first degree relatives, behaved in the same way with regard to arachidonic acid metabolism.

Benoxaprofen, when used *in vitro* at a concentration similar to the level of free drug achieved in the plasma in patients on a standard dose regimen, diminished the levels of 12,20-diHETE. This is of interest in view of the beneficial effect of benoxaprofen in psoriasis, and raises further questions about the mode of action of the drug. In common with other studies (Salmon *et al.*, 1984; Cashin, Dawson & Kitchen, 1977) we have shown that benoxaprofen has some cyclo-oxygenase inhibitory activity. The increase in 12-HETE observed may be a result of substrate diversion or due to a direct stimulatory effect of the drug on 12-lipoxygenase. Harvey *et al.* (1983), using a preparation of human platelet microsomes, found that benoxaprofen did not inhibit the formation of 12-HETE, and no stimulatory effect was mentioned. Indomethacin, which appears to exacerbate rather than ameliorate psoriasis (Katayama & Kawada, 1981; Ellis *et al.*, 1983), had a similar *in vitro* effect to benoxaprofen on 12-HETE and cyclo-oxygenase products, but differed in having no inhibitory effect on 12,20-diHETE production.

We also found that production of $TXB_2$ by mixed cells from psoriatics was diminished, when corrected for the number of platelets present. HHT, another platelet metabolite, production of which normally occurs simultaneously with that of $TXB_2$, was also found to be formed in reduced amounts, even without correcting for the platelet count. Fallon & Kragballe (1984) studied arachidonic acid metabolism in purified platelets stimulated by thrombin, in the absence of white cells, and found that $TXB_2$, HHT and 12-HETE were all generated in increased amounts in psoriatic patients. There is, however, some evidence for inhibition of the cyclo-oxygenase pathway in psoriasis; Penneys *et al.* (1975) found an inhibitor of $PGE_2$ synthesis in psoriatic plaque. It is possible that a similar inhibition exists in the circulating blood cells, manifesting itself as a decrease in $TXB_2$ and HHT production by platelets. The effect may be slight, and demonstrable only in the presence of white cells when there is competition and possibly transfer of available substrate between cells (Marcus *et al.*, 1984). We are currently attempting to clarify whether there is a primary abnormality of arachidonic acid metabolism in psoriatic platelets.

Because of the complex relationship between platelet count and 12-HETE production, it is difficult to draw any conclusions about the latter from the present study; this information should emerge from studies on isolated platelets. It will be important to measure 12-HETE production from psoriatic platelets, in view of our finding of elevated production of 12,20-diHETE (which is derived from 12-HETE) in preparations of peripheral blood cells from psoriatic subjects, and the original observation by Hammarstrom *et al.* (1975) of raised 12-HETE levels in psoriatic epidermis.

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# Complement cleavage products in the phototoxic reaction of porphyria cutanea tarda

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## SUMMARY

We have measured C3, C4, CH50 and complement cleavage products C3a and C5a in sera and plasma from PCT patients and normal controls 10 min and 1, 4 and 24 h after UVA irradiation.

We found elevated C3a concentrations in PCT patients immediately after UVA irradiation and 24 h later. The same was true for CH50, whereas C3, C4 and C5a did not change significantly. No such changes occurred in normal controls.

Our data suggest that activation of the complement cleavage product C3a by porphyrin and UV light triggers a series of events that cause tissue damage.

Porphyria cutanea tarda (PCT) is a metabolic disease, usually acquired, with skin photosensitivity associated with an excess of uroporphyrin in blood and urine.

It has been shown that irradiation at 400–410 nm, of sera from patients with erythropoietic protoporphyria and porphyria cutanea tarda resulted in decreased haemolytic titres of some complement factors (Lim, Poh-Fitzpatrick & Gigli, 1984; Lim, *et al.*, 1981; Lim & Gigli, 1981). This also occurs *in vivo* after irradiation of the ventral and dorsal skin surface of the forearms of patients with PCT.

We have recently shown that factors activated by porphyrin and UVA light stimulate neutrophilic granulocytes to produce superoxide, triggering a series of events that enhances tissue damage. We also found activation of donor polymorphonuclear leukocytes (PMN) by porphyrin without addition of zymosan to the sera. (Pigatto, *et al.*, 1985). We proposed therefore that the complement cascade might be an autologous PMN activation. These data and the selective involvement of granulocyte cells known to be reactive to activated complement components suggested that complement cleavage products might be directly involved in the phototoxic reaction in PCT. We have investigated the role of C3a and C5a to determine whether UVA irradiation *in vivo* is able to induce complement activation, and whether one or more of the

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low molecular weight activated components participates in the porphyrin-induced phototoxicity.

## METHODS

### *Patients*

Seven men aged 44–62 years (mean 50 years) with active PCT were studied. All the patients were hospitalized and protected from ultraviolet light for at least 15 days. Diagnoses were based on the histories, objective criteria and determination of the pattern of urinary porphyrins by HPLC, as described by Doss (1970), and blood porphyrins as described by Topi, Amantea and Griso (1984).

Liver involvement was assessed by tests for serum and urine bilirubin, urine urobilinogen, serum enzymes, serum proteins, blood lipids, and immunological tests for smooth muscle and antimitochondrial antibodies. All the patients had liver disorders, and needle biopsies were taken by the Menghini technique for histological evaluation according to the criteria of Groote (1968). The histology ranged from active chronic hepatitis to cirrhosis.

### *Controls*

Ten matched controls were drawn from the hospital staff. The same blood tests were taken, but no liver biopsies.

### *Phototesting*

All subjects were phototested to determine the minimal erythemal doses (MED) of UVA exposure for production of detectable erythema with definite margins, 48 or 72 h after exposure. The lower part of the back or the buttocks were used. MEDs ranged from 1 to 5 J/cm$^2$ for the PCT patients and from 10 to 25 J/cm$^2$ for the controls.

### *Irradiation*

All the patients and the controls were irradiated over the entire body surface to give a dose corresponding to 75% of the MED with a radiation exposure device (Blue Light 2005·1 Dr Mönle GmbH, München, F.R.G.) that contained five metal halide (Osram ultramed) lamps, with an $H_1$ filter (Schott Janerglass-Werke; Mainz, F.R.G.), to obtain the UVA spectrum (315–410 nm).

Venous blood samples were obtained before irradiation, and at 10 min and 1, 4 and 24 h after irradiation. Each 20 ml blood sample was divided into three aliquots, one of which was mixed with EDTA, one with heparin (Liquemin, Roche), and one allowed to clot for 1 h at room temperature and placed in ice to obtain maximal retraction of the clot. All procedures after the blood was drawn were carried out in a dark room under a red light.

### *Complement assays*

Complement (C3 and C4) was measured by radial immunodiffusion (Behring Diagnostics, Sommerville, NJ, U.S.A.) and levels expressed in mg/dl. The serum haemolytic titre of CH50 was measured by the method of Olitzky (1968) and expressed as units/dl. Radioimmunoassays for C5a desarginine and C3a desarginine (using kits from Upjohn Company, Michigan, U.S.A.) were performed by the method of Hugli and Chenoweth (1980).

### *Chemotaxis assays*

Polymorphonuclear leukocytes from normal control subjects and PCT patients were isolated by a modification of the method of Maderazo and Woronick (1978). The PMNs were adjusted to a concentration of $2 \times 10^6$ cells per ml/medium. The final cell preparation contained more than 90% neutrophils, with viability greater than 95% as assessed by trypan blue exclusion. Normal human serum was incubated with 5 mg/ml zymosan (Sigma Chemical Co.) in a 37°C water bath for 30 min. The activated serum (ZAS) was centrifuged for 10 min at 700 ***g*** and stored in liquid nitrogen. For assays, ZAS serum was diluted 1:200 with RPMI 1640. RPMI 1640 plus FMLP at a concentration of $10^{-9}$ mol/l was used as a second chemotactic factor.

Chemotaxis was tested *in vitro* by the Boyden method, modified by Siccardi, as previously described (Pigatto *et al.*, 1983). A two-compartment chamber (LP Italiana S.p.A., Milan, Italy) was used, with the compartments separated by a Millipore filter with 3-μm pores. The PMN suspension was placed in the upper compartment and RPMI 1640 with or without ZAS and FMLP in the lower compartment. The chambers were incubated for 1·5 h at 37°C. The filters were removed, fixed in methanol, stained with haematoxylin, and mounted on glass slides. All assays were run in duplicate. To measure leukocyte mobility, the 'leading front' technique was used, and the results, expressed in μm, are the differences between chemotactic and random migration in the distance covered by the three fastest cells in 10 microscopic fields.

### *Statistical analysis*

The results were analysed using Student's *t*-test for paired and unpaired data.

## RESULTS

Plasma levels of C3a were elevated significantly before irradiation in PCT patients compared with normal controls ($P < 0{\cdot}01$) and they continued to increase steadily thereafter (Fig. 1a). At the end of the experiment mean C3a levels were more than twice as high as pre-radiation levels (Fig. 1a). C3a levels did not change in normal controls after irradiation.

Plasma CH50 haemolytic titres of normal subjects and PCT patients were not significantly different before irradiation and had not changed significantly 10 min after irradiation (Table 1). There was a significant difference in CH50 between the two groups at 1, 4 and 24 h after irradiation ($P < 0{\cdot}05$). Plasma levels of C3 and C4 did not change significantly in either group (Table 1); nor did C5a levels in either group (Fig. 1b).

Before UVA irradiation, PMN from PCT patients and normal controls showed a similar chemotactic response to ZAS and to FMLP, but at 10 min and at 1 and 4 h after irradiation chemotaxis toward ZAS was significantly reduced ($P < 0{\cdot}01$), whereas the chemotaxis to FMLP was essentially unchanged. 24 h after irradiation, the chemotaxis toward ZAS was less than that before irradiation but the difference was not significant (Table 2).

## DISCUSSION

Irrespective of skin manifestations, complement activation was clearly demonstrable in the PCT patients after UVA irradiation. We also observed significantly elevated plasma levels of C3a before UVA radiation. This is due probably to some complement activation by porphyrin and UVA that patients may have been exposed to during their stay in the hospital, although we protected them from ultraviolet light as much as possible for at least 15 days. In all seven patients, further increases in plasma levels of C3a were observed 10 min after irradiation. Therefore, complement activation appears to be a phenomenon that occurs in all patients.

These findings corroborate our earlier reports and those of other workers of *in vitro* and *in vivo* activation of complement, and suggest an association between the anaphylatoxins C3a and C5a

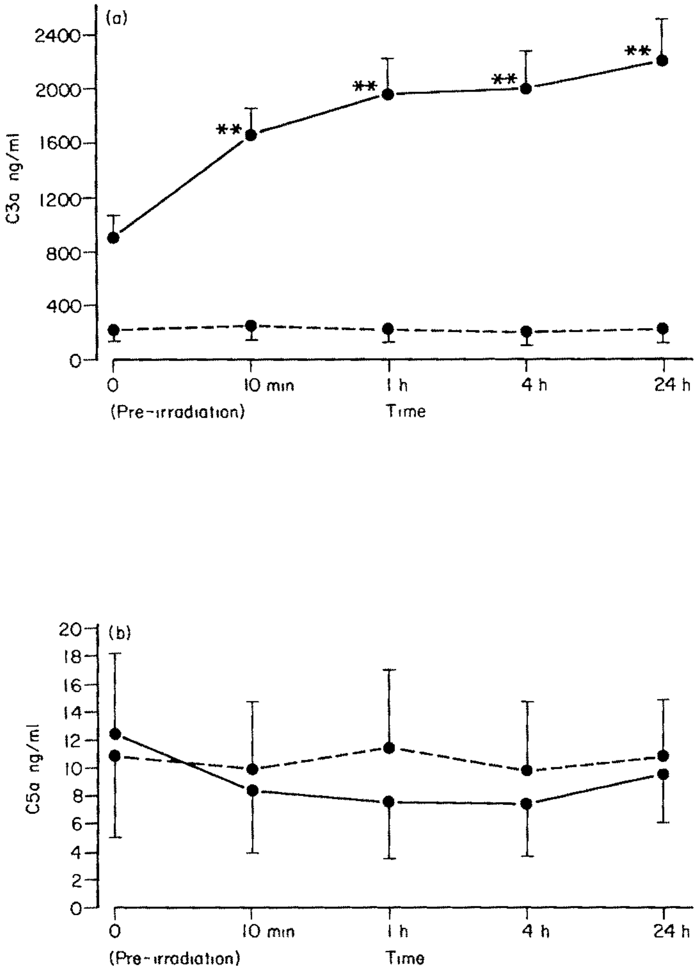

FIGURE 1. Plasma levels of (a) C3a and (b) C5a in patients (——) and normal controls (– – – –) undergoing UVA irradiation. Points represent mean ± SD for seven patients or 10 controls. Comparison with controls $^{**}P < 0.001$.

and porphyrin-induced phototoxicity (Kamide, Gigli & Lim, 1984). We have demonstrated also that CH50 haemolytic titre was decreased in PCT 1 h after irradiation. The reason for this difference in timing may be that the radioimmunoassay for C3a is a more sensitive method for detecting complement conversion *in vivo*. In our experiment, C5a had not changed significantly at any time point.

However, our failure to detect an increase in free plasma C5a after UVA irradiation, in patients with PCT, is in agreement with the observations of other investigators (Craddock *et al.*, 1977; Chenoweth *et al.*, 1981). C5a, a factor that binds avidly to neutrophils (Chenoweth & Hugli, 1978), is detectable in serum only in the few moments following activation. For this reason, we studied the chemotactic activities of PMN from patients and controls. We used ZAS and nFMLP because they are of approximately equal potency, and investigated the responses of two different PMN receptors. After their exposure to C5a, neutrophils are unable to react again

TABLE 1. Complement (CH50, C3, C4) levels before and after UVA irradiation in patients with PCT and normal controls

| Subjects | Time of measurement | CH50 Haemolytic units/dl (mean ± SD) | C3 mg/dl (mean ± SD) | C4 mg/dl (mean ± SD) |
|---|---|---|---|---|
| PCT ($n = 7$) | Pre-irradiation | 1350 ± 239 | 98 ± 33 | 17·5 ± 5·9 |
| | Post-irradiation: | | | |
| | 10 min | 1460 ± 268 | 99 ± 34 | 16 5 ± 4 6 |
| | 1 h | 872 ± 309* | 81 ± 28 | 18 2 ± 7 2 |
| | 4 h | 830 ± 340* | 88 ± 25 | 17·2 ± 4 8 |
| | 24 h | 910 ± 210* | 92 ± 31 | 18·3 ± 5·0 |
| Normal controls ($n = 10$) | Pre-irradiation | 1412 ± 220 | 95·2 ± 15 | 17 3 ± 2 6 |
| | Post-irradiation: | | | |
| | 10 min | 1530 ± 310 | 103 ± 34 | 17 4 ± 4 2 |
| | 1 h | 1380 ± 120 | 102 ± 26 | 18 1 ± 5 9 |
| | 4 h | 1284 ± 184 | 94 ± 28 | 18·0 ± 4·0 |
| | 24 h | 1341 ± 212 | 94·8 ± 26 | 17·8 ± 6·3 |

* Significantly different from pre-irradiation levels and from levels in control subjects ($P < 0·05$).

TABLE 2. Chemotactic responses to zymosan-activated sera (ZAS) and FMLP of PMN from seven PCT patients and 10 normal subjects before and after UVA irradiation

| Time of measurement | ZAS | | FMLP | |
|---|---|---|---|---|
| | PCT | Normal | PCT | Normal |
| Pre-irradiation | 58 ± 10 | 61 ± 8 | 62 ± 10 | 64 ± 12 |
| Post-irradiation: | | | | |
| 10 min | 33 ± 14* | 60 ± 9 | 68 ± 8 | 67 ± 10 |
| 1 h | 36 ± 12* | 58 ± 11 | 61 ± 11 | 65 ± 8 |
| 4 h | 39 ± 10* | 59 ± 7 | 64 ± 10 | 67 ± 11 |
| 24 h | 54 ± 11 | 61 ± 11 | 65 ± 13 | 63 ± 9 |

* Significantly different from pre-irradiation value and from control ($P < 0·01$).

to this factor (found in ZAS) for 4 h, but they still react to FMLP. The selectivity of the time-dependent loss of responsiveness in these cells suggests that their deactivation may result from receptor internalization (Chenoweth *et al.*, 1981). Although we have not been able to demonstrate directly an increase in the C5a generated by UVA plus porphyrin in sera, our data suggest that neutrophils may be able to capture C5a as soon as it is generated.

In a few diseases there is such a high level of complement activation that it exceeds the neutrophil C5a binding capacity (Hammerschmid *et al.*, 1980). In PCT, as in other pathological conditions, there is no abnormality of the C5a clearance mechanism, although C5a is produced in excess.

In a previous study (Pigatto *et al.*, 1985) we found that serum from irradiated PCT patients generated significantly greater amounts of superoxides from PMN of normal donors than did irradiated serum from normal controls. Our previous and current data suggest that complement cleavage products activated by porphyrin and by UV light stimulate neutrophilic granulocytes, triggering a series of events that leads to the neutrophil infiltration observed in the delayed phase of the reaction. The present study clearly demonstrates that UVA irradiation of PCT patients promotes formation of C3a anaphylatoxin. The fact that complement activation occurs after UV irradiation in PCT patients does not prove that anaphylatoxins are responsible for the clinical features. They could simply be an associated abnormality. The results of complement activation (Lim *et al.*, 1984; Lim *et al.*, 1981; Lim & Gigli, 1981), mast-cell degranulation (Kamide *et al.*, 1984), generation of reactive oxygen products (Pigatto *et al.*, 1985), arachidonic acid metabolites (Lord *et al.*, 1976) and neutrophil activation can lead to severe tissue damage (Allison, Magnus & Young, 1966).

To the extent that complement cleavage products are responsible for the pathophysiological sequelae of the porphyrin toxic reaction, these observations may lead to some means of controlling this damaging skin disease.

## ACKNOWLEDGMENT

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# Recurrent viral infections in patients with past or present atopic dermatitis

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## SUMMARY

Histories of recurrent infections were obtained from 955 adults with past or present atopic dermatitis (AD) and from 199 controls. Patients who had previously been hospitalized in childhood because of severe AD had a significantly higher incidence of recurrent (> 5 episodes per year) cold sores and upper respiratory tract infections as well as a higher incidence of herpes zoster, than non-atopic controls. Patients with milder AD in childhood (never hospitalized) had lower incidence of these diseases, but as regards cold sores, the frequency of recurrent infections was still significantly higher than that of the controls. AD patients with past or present respiratory allergy had a slight but not statistically significant increased incidence of recurrent infections, as compared to AD patients without concomitant respiratory allergy. The factors predisposing to frequent infections appeared to be those related to severity of the atopic condition, i.e. ongoing AD, need for hospitalization in childhood and extent of dermatitis. The increased susceptibility to recurrent viral infections in AD patients is most probably related to dysfunctioning cell-mediated immunity.

Atopic dermatitis (AD) is a disease that has been associated with multiple abnormalities of both the cell-mediated and humoral branches of the immune system (Hanifin & Lobitz, 1977; Strannegard & Strannegard, 1978). Defective function of cell-mediated immunity in AD, which may be due to dysfunction of T cells (Strannegard & Strannegard, 1978), macrophages (Kragballe, 1979; Strannegard, 1981), interferon production (Strannegard & Strannegard, 1980), or decreased NK cell or K cell activity (Jensen *et al.*, 1984), has been thought to predispose to cutaneous viral infections of unusual severity, in particular vaccinia and herpes simplex infections, although it has not been proven that defective immunity rather than cutaneous alterations is the predisposing factor. In earlier studies (Strannegard & Strannegard,

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1981; Rystedt, Strannegard & Strannegard, 1984) we have found evidence for an unusual response to EB virus infections in AD, but no serological evidence for increased occurrence of, or abnormal response to, any other types of viral infections.

Previous clinical studies of viral infections in AD patients have dealt mainly with the well-known syndromes of eczema vaccinatum and eczema herpeticum. In the present study we have attempted to determine the incidence of two viral infections, herpes zoster and herpes simplex, and also of upper respiratory tract infections which are almost always due to viruses. Both herpes zoster and recurrence of herpes simplex appear to be associated with depression of cell-mediated immunity (Rand *et al.*, 1977) and frequent upper respiratory tract infections have been noted in children with a defective interferon response (Isaacs *et al.*, 1981). In view of the defective cell-mediated immunity in AD, it might be expected that patients with this disease should have an increased incidence of recurrent viral infections.

## PATIENTS

Three groups of patients participated in the study.

### *Group I*

Group I comprised 549 individuals, 290 men and 259 women, who during the period 1952–56 had been admitted at least once to the Department of Dermatology, Karolinska Hospital, Stockholm, Sweden, and at that time had been allotted the diagnostic number for AD as used at the hospital during those years. Patients considered to have the seborrhoeic form of infantile eczema on admission to hospital were not included. The group originally comprised 610 people, *viz* every patient aged 0–14 years treated at the Department during those years. Six who did not fulfil the cirteria for AD set up by Hanifin and Rajka (1980) were excluded from the follow-up after scrutiny of the records or after interview. Of the remainder, 30 had emigrated, nine had died, eight could not be traced owing to faulty recording of personal data, and eight failed to reply to repeated letters and had no telephone. None of the deaths was due to allergic disease. Thus, 99% of those who could be reached took part in the study.

### *Group II*

Group II comprised 406 individuals, 217 men and 189 women, who had attended the out-patient clinic at the Department of Dermatology during the period 1955–56 but had not been admitted to hospital. Other criteria for inclusion in the study were the same as for Group I.

During these years 460 patients had been assigned the diagnositiç number for AD. Checking of out-patient records and interviews with the patients showed that 24 diagnoses turned out not to fulfil the Hanifin and Rajka (1980) criteria, in many cases owing to incorrect recording of the diagnostic number. Of the 436 people invited for interview, nine had emigrated, four had died but not from allergic disease, two were in psychiatric hospitals, four could not be traced owing to wrong recording of personal data, and 11 failed to reply to repeated letters and had no telephone. Thus, 97% of those who could be reached were interviewed.

### *Group III*

Group III comprised 199 individuals, 114 men and 85 women, with no history or current symptoms or signs of atopic disease and no family history of atopy in parents, grandparents, siblings, uncles or aunts. The group, which originally comprised 461 individuals, was selected at random by the data processing company DAFA, Stockholm, from the Swedish population register, after matching against people in Groups I and II with respect to sex, age, nationality and place of domicile (Stockholm County or other district). Three had emigrated, one had died, seven had moved, and five did not wish to participate. The 445 interviewed thus constituted 99% of those who could be reached. All were questioned in detail, and 246 with past or present atopy or a history of atopy in parents, siblings, or distant relatives were then excluded using very strict criteria. Thus, individuals with any suspicion of familial or personal atopy, i.e. AD, bronchial asthma, allergic rhinitis, possible gastro-intestinal allergy and urticaria, were excluded.

## METHODS

A detailed questionnaire with 142 questions regarding duration of AD, localization of AD in childhood and in adult life, family atopy, associated respiratory allergy and occurrence of infections was used as a basis for interviewing all individuals participating in the study. In addition, 183 randomly-chosen patients in Group I and 162 patients in Group II were clinically examined at the time of the inverview. All subjects were interviewed either personally by one of us (IR) or by telephone.

Cold sores were scored (i) never; (ii) seldom; (iii) 2–5 times/year; (iv) more than 5 times/year. Upper respiratory tract infections (URTI) were scored (i) seldom; (ii) 2–5 times/year (iii) more than 5 times/year. In both cases only the last score (more than 5 times/year) was included in the analysis of the results.

IgE determinations were performed on sera drawn from the individuals in Groups I and II who were randomly chosen for clinical investigation. The determinations were performed with commercially available PRIST (R) reagents (Pharmacia, Uppsala, Sweden) following the recommendations of the manufacturer. The results were expressed as units/ml in accordance with WHO recommendations.

All statistical comparisons were made using a chi square test.

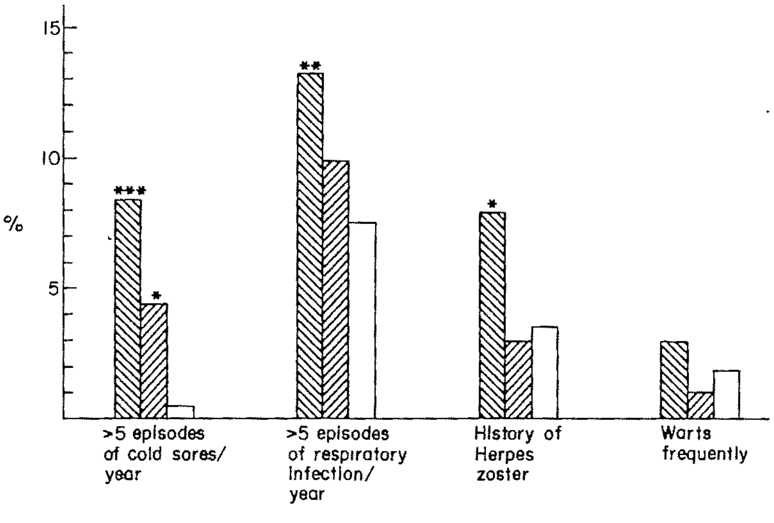

FIGURE 1. History of cold sores, respiratory infections, herpes zoster and warts in patients with atopic dermatitis and in normal controls. Comparison with controls $^{*}P<0{\cdot}05$; $^{**}P<0{\cdot}01$; $^{***}P<0{\cdot}001$. ▧ previously hospitalized AD patients, $n=549$; ▨ out-patients with AD, $n=406$; □ controls, $n=199$.

## RESULTS

### *History of cold sores, herpes zoster, upper respiratory infections and warts in patients with atopic dermatitis*

A larger proportion of individuals who had been hospitalized previously for AD (Group I) gave histories of frequent cold sores and URTI than did the out-patients (Group II) and the controls (Group III) (Fig. 1). The differences were most marked with respect to frequent cold sores, which were reported by 8·4% of Group I and by 4·4% of Group II compared with 0·5% in the controls ($P < 0{\cdot}001$ and $P < 0{\cdot}05$ respectively). Histories of frequent URTI were significantly more common in Group I than in the controls ($P < 0{\cdot}01$). A similar result was found for Group II, although this difference was not statistically significant.

Group I patients gave more frequent histories of herpes zoster than did the controls ($P < 0{\cdot}05$). Histories of frequent herpes zoster infection in Group II were not significantly more common than in the controls.

Identical questionnaires regarding occurrence of warts were used for Groups II and III, while Group I patients filled in a different form. Therefore, the proportion in Group I was not strictly comparable with those in Group II and III. However, there was no indication that Group I patients had an increased incidence of warts and there was no statistically significant difference in the incidence of warts between Group II and III.

### *Relation between frequent infections and activity of AD*

As seen in Table 1, patients with ongoing AD had the highest incidence of histories of frequent cold sores, URTIs, and herpes zoster. There was a clear relationship between activity of dermatitis and the occurrence of frequent infections; the longer the time of remission, the lower the incidence of frequent infections. This pattern was seen for all the AD patients but was particularly evident in Group I (Fig. 2). Even individuals who had not had any signs of AD during the year preceding the interview had a significantly higher frequency of recurrent herpes

TABLE 1. Relationship between frequent infections and activity of atopic dermatitis

| | | % of subjects† | | |
|---|---|---|---|---|
| Category | No. of subjects | Frequent cold sores | Frequent URTI | History of zoster |
| Controls | 199 | 0·5 | 7·5 | 3·5 |
| No AD during preceding 10 years | 228 | 2·6 | 9·2 | 2·6 |
| No AD during preceding year | 351 | 4·6** | 9·4 | 4·3 |
| No AD at interview | 453 | 6·0** | 10·6 | 4·4 |
| Ongoing AD | 502 | 7·4** | 12·9* | 7·0 |

† Results for Groups I and II were pooled.
Comparison with controls *$P < 0{\cdot}05$ **$P < 0{\cdot}01$.

TABLE 2. Relationship between frequent infections and localization of dermatitis

| Localization | | No. of patients | % of patients<br>Frequent cold sores | <br>Frequent URTI | <br>History of zoster |
|---|---|---|---|---|---|
| Body | Group I | 386 | 9·8 | 15·5 | 8·5 |
| Body | Group II | 217 | 5·5 | 11·1 | 2·3 |
| Hand | Group I | 67 | 7·5 | 7·5 | 10·4 |
| Hand | Group II | 57 | 5·3 | 5·3 | 7·0 |
| None* | Group I | 96 | 3·1 | 8·3 | 3·1 |
| None* | Group II | 132 | 2·3 | 9·8 | 2·3 |

* No signs of AD for at least 10 years.

simplex infections than the controls ($P<0{\cdot}01$). In these 'healed' patients there was also a tendency towards higher incidence of recurrent herpes zoster infections and URTI than in the controls.

### *Relation between frequent infections and localization of AD*

Individuals in Groups I and II were sub-divided according to the localization of their dermatitis, to the hands only, or to the body with or without hand dermatitis. As shown in Table 2, cold sores and frequent URTI were more prevalent in patients with body dermatitis than in those with hand dermatitis only. The highest incidence of frequent cold sores and URTI was

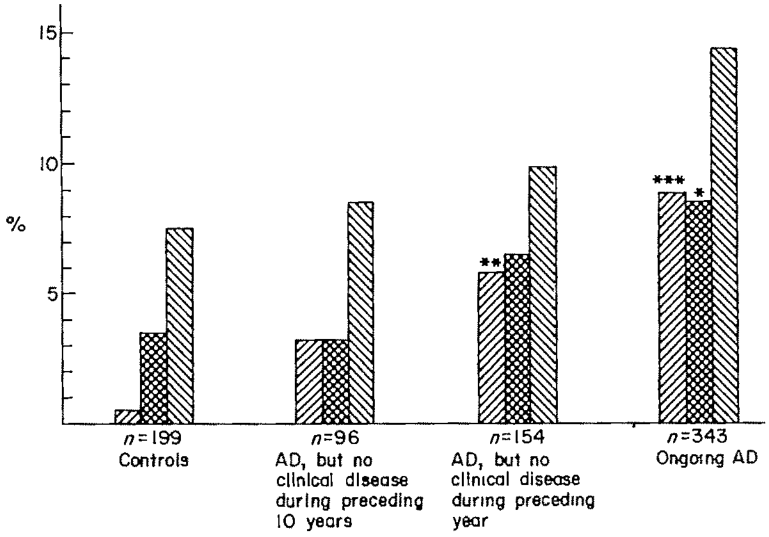

FIGURE 2. Frequency of infections in previously hospitalized AD patients (Group I) in relation to the activity of their dermatitis. Comparison with controls $^{*}P<0{\cdot}05$; $^{**}P<0{\cdot}01$; $^{***}P<0{\cdot}001$. ▨ > 5 episodes of cold sores/year; ■ history of zoster; ▧ > 5 episodes of URTI/year.

TABLE 3 Incidence of infections in AD patients with or without respiratory allergy and raised IgE levels

| | No. of subjects | % of subjects | | |
|---|---|---|---|---|
| | | Frequent cold sores | Frequent URTI | History of zoster |
| Previous or present AD; never respiratory allergy | 339 | 5·6** | 9·4 | 5·3 |
| Previous or present AD; previous or present respiratory allergy | 616 | 7·3*** | 13·1* | 6·0 |
| Serum IgE <200 U/ml | 194 | 7·2 | 9·3 | 7·8 |
| Serum IgE >200 U/ml | 115 | 7·0 | 13·9 | 7·8 |
| Controls | 199 | 0·5 | 7·5 | 3·5 |

Comparison with controls $^{*}P<0{\cdot}05$; $^{**}P<0{\cdot}01$; $^{***}P<0{\cdot}001$.

observed in Group I patients with body dermatitis. The reverse was found with herpes zoster infection which was more common in the hand dermatitis group. None of these differences, however, was statistically significant.

### *Incidence of infections in AD patients in relation to concomitant respiratory allergy and raised serum IgE levels*

As shown in Table 3, an increased incidence of infections was found in AD patients, irrespective of whether they had accompanying respiratory allergy. There was a slightly higher incidence of frequent URTI in patients with past or present respiratory allergy (13·1%) than in those without respiratory allergy (9·4%) but the difference did not quite reach statistical significance ($P=0{\cdot}054$).

No significant differences in cold sores and herpes zoster were found between patients with and those without respiratory allergy.

There was no obvious relationship between serum IgE levels and occurrence of infections. However, IgE was not determined in all patients and some of the results were derived from small groups only.

## DISCUSSION

Patients with AD are known to exhibit an unusual susceptibility to severe cutaneous infections. Kaposi (1895) observed that herpes simplex and vaccinia virus infections may run a very severe course in these patients. Increased susceptibility to warts, molluscum contagiosum, fungal and staphylococcal infections has also been observed in AD (Currie, Wright & Miller, 1971; Solomon & Telner, 1966; Hanifin, Ray & Lobitz, 1974; Hanifin & Lobitz, 1977). The reason for this increased susceptibility to infections has not been clearly established but it has often been considered that cutaneous alterations are responsible, since there has been no demonstration of heightened susceptibility to infections that do not involve the skin. However, the recent demonstration of defective cell-mediated immunity in AD, involving T-cell dysfunction (Strannegard, Lindholm & Strannegard, 1976; Strannegard & Strannegard, 1978) suggests that immunological dysfunction may predispose these patients to the severe infections observed.

From the finding of T-cell dysfunction, we predicted that AD patients should suffer from an increased incidence of recurrent viral infections, since the host defence against such infections appears to be mainly dependent on cell-mediated immunity. The present study has validated this prediction. Patients with AD did indeed give histories of an increased frequency of cold sores, as well as herpes zoster and upper respiratory tract infections. Thus, the incidence of two recurrent viral infections (cold sores and herpes zoster), and one infection almost always of viral origin (URTI) was higher in AD patients.

Of particular interest was the finding of an increased incidence of recurrent cold sores also in patients without signs of ongoing dermatitis. This would suggest that increased susceptibility to recurrent viral infection is not necessarily dependent on cutaneous alterations, but rather reflects basic immunological abnormalities. The finding of a slightly increased incidence of URTI also in AD patients without concomitant respiratory allergy supports this suggestion.

The defence mechanisms against recurrent herpes virus infections are not fully understood, but are believed to be primarily dependent on cell-mediated immunity. Recently, Cunningham and Merigan (1983) found a correlation between the time interval between episodes of recurrent herpes labialis and the level of interferon-gamma (IFN-$\gamma$) production. This suggests that cell-mediated immunity, operating at least partly through interferon secretion, is important for the maintenance of herpes virus latency. This suggestion is supported by animal experiments (Openshaw *et al.*, 1980) and the finding of herpes virus infections in immunosuppressed transplant patients (Rand *et al.*, 1977). Therefore, patients with defective cell-mediated immunity, perhaps, in particular, defective interferon production, would be expected to be prone to recurrent herpes virus infections. Thus, it is not surprising that we found an increased occurrence of recurrent herpes simplex and herpes zoster infections in AD patients, who have been shown to have deficient cell-mediated immunity (Hanifin & Lobitz, 1977; Strannegard & Strannegard, 1978). Previously, we have found defective interferon-alfa production in a group of atopic children (Strannegard & Strannegard, 1980) but the production of IFN-$\gamma$ in AD has to our knowledge, not been extensively studied. However, the mitogenic response of T-lymphocytes, which are known to produce IFN-$\gamma$, is decreased in AD (Strannegard *et al.*, 1976), as is the production of lymphocyte migration inhibitory factor (Horsmanheimo & Horsmanheimo, 1979). Therefore, there is reason to suspect that IFN-$\gamma$ production may be impaired in AD.

Not only herpes virus infections but also URTI may be related to an abnormality in the IFN-producing system. Recently, Isaacs *et al.* (1981) found deficient IFN-$\alpha$ production in some children with recurrent respiratory tract infections. This finding fits with the previous demonstration of impaired IFN-$\alpha$ production in atopy (Strannegard & Strannegard, 1980) and the present results showing an increased rate of URTI in atopic dermatitis. It might be argued that increased susceptibility to respiratory infections is a specific feature of respiratory allergy and not related to AD. However, we found a tendency towards increased rates of respiratory infections also in AD patients without any history of respiratory allergy or increased IgE levels. In previous studies it has not been clearly shown that respiratory allergy is associated with increased susceptibility to respiratory infections (Minor *et al.*, 1974; Isaacs *et al.*, 1982). Therefore, we suggest that the significant association between AD and an increased rate of respiratory infections is not solely attributable to the respiratory allergy that frequently occurs with the dermatitis, but, rather, reflects the underlying immunological abnormality in the patients.

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# A serological mediator in chronic idiopathic urticaria—a clinical, immunological and histological evaluation

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## SUMMARY

Seven of twelve autologous sera from patients with chronic idiopathic urticaria re-injected intradermally produced a weal at the site of injection. There was no response in 19 control subjects. Patients showing a positive response had a shorter duration of disease and shorter duration of spontaneous weals, and their urticaria was less likely to be exacerbated by pressure. There was some serological evidence of circulating immune complexes in both positive and negative responders to autologous serum, but only two showed complement abnormalities. When six of the serum-positive patients were re-tested after one year, five still showed a positive response with their original stored serum, but only two, whose disease remained active, were positive when challenged with freshly drawn serum, suggesting that a serum mediator is only present when the urticaria is active. A marked neutrophil infiltrate was seen within and around small dermal blood vessels at the injection site in the majority of urticaria patients but this appearance did not correlate with weal formation. In control subjects the cellular response was mild and mainly mononuclear.

The aetiology of chronic urticaria is poorly understood. Studies by Monroe *et al.* (1981) and Russell Jones *et al.* (1983) have shown a spectrum of histological changes ranging from mild perivascular infiltrates, through dense mixed mononuclear or neutrophil infiltrates, to overt necrotizing vasculitis with leukocytoclasis, fibrinoid changes in the vessel walls and erythrocyte extravasation. Many of the patients with histological features of necrotizing vasculitis showed evidence both of immune complexes in the circulation and of complement consumption. Some also had cryoglobulinaemia.

Whitsed & Penny (1971) showed that autologous plasma and isolated cryoglobulins

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re-injected intradermally produced vasculitic lesions in a patient with vasculitis and cryoglobulinaemia. In preliminary studies we noted that some patients with chronic urticaria also produce a weal at the site of re-injection of their own serum, suggesting that active material is present in the circulation and can be detected through its effect on the skin, even though it may not be evident in serological tests. We have attempted to define this response clinically and histologically in patients with chronic urticaria, using parallel serological tests for immune complex formation, complement activation and cryoglobulin production to attempt to correlate these with the skin test response. Skin testing was repeated after one year to establish whether skin test reactivity altered with changing disease activity.

## METHODS

Twelve patients (four male, eight female) with chronic idiopathic urticaria of more than six weeks' duration presenting to clinics at the Bristol Royal Infirmary were studied. All gave written informed consent. Patients less than 16 years old and those with symptomatic dermographism or other predominantly physical urticaria were excluded. Patients' mean age at presentation was 46 years (range 24–78). Nineteen healthy control subjects (9 male, 10 female; mean age 40·8 years, range 20–81) were also skin tested with their own serum and nine were biopsied. The urticaria patients were admitted overnight after stopping antihistamine therapy for at least 48 h. A full history and examination were undertaken, with particular reference to evidence of atopy, vasculitis or connective tissue disease.

Blood was taken for full blood count, plasma viscosity, immunoglobulins A, G and M, IgE (PRIST, Pharmacia), immunoelectrophoresis, liver function tests, $HB_sAg$, cryoglobulins, standard autoimmune profile, C3, C4, CH50, alternative pathway and immune complex tests which included fluid phase C1q binding, anti-complement activity (ACA), platelet aggregation titre (PAT) and quantitation of protein precipitated by 2% polyethylene glycol 6000 (PEG) (Haeney, 1981).

Blood from both patients and controls was allowed to clot for 30 min in sterile glass bottles and centrifuged at room temperature. Serum was withdrawn using aseptic technique and one or more 0·1 ml aliquots re-injected intradermally into volar forearm skin with a 27 gauge needle, the remainder being stored at −20°C. Sterile normal saline was injected into the other forearm at the same time as a within-patient control.

Biopsies were taken from paired serum and saline skin tests in all the patients and nine of the 19 controls between 90 and 135 min from the time of injection, using a 4 mm punch under lignocaine with adrenaline local anaesthesia. Specimens were divided, half being fixed in 10% formal saline for routine paraffin processing and half snap frozen in liquid nitrogen for direct immunofluorescence using fluorescein isothiocyanate labelled antisera to IgG, A, M, C3 and fibrin (Dako Laboratories, High Wycombe, Berkshire, U.K.).

Histology was assessed by two independent observers without reference to the subjects' identity or group. The quantity of cellular infiltration was graded into three categories, mild, moderate or marked, and the predominant cell type was recorded as neutrophil predominant, mononuclear cell predominant or mixed. The presence of eosinophils and distribution of the infiltrate was also noted.

Skin tests were repeated in each patient with the same serum, and using serum drawn during varying phases of urticarial activity. Measurements of weal size and the surrounding erythema were made immediately after injection, after 1 h and after 2 h and at variable intervals for up to 24 h, measuring only skin tests that had not been biopsied. The maximum and minimum diameters of both weal and confluent erythema were measured with a millimeter scale and their means recorded.

Patients were classified as serum positive if their own serum produced a weal within 2 h of intradermal re-injection, provided that the weal size was at least 5 mm greater than the saline control and there was a difference of at least 10 mm in the diameter of the surrounding erythema ($W \geqslant C+5$ and $E \geqslant C+10$). The skin tests were repeated one year later in six of the subjects who were serum positive, using the patient's fresh serum and also the serum that had been stored from the original skin test.

## RESULTS

### *Skin tests and clinical characteristics*

Seven of the 12 patients had a positive skin test. Their main clinical characteristics are summarized in Table 1, which shows that the serum-positive patients had a shorter duration of disease and shorter duration of spontaneous weals, and were less likely to give a history of exacerbation with pressure. None of the 19 control subjects had a positive skin test.

The effects of re-injection with serum drawn at different phases of urticarial activity and at follow-up one year later are shown in Table 2. This demonstrates that the skin test remains positive irrespective of the activity of urticaria when the serum is drawn and it only converts to negative as the disease remits. Similarly, in the serum-negative group the skin tests remained consistently negative whether serum was drawn during an eruption of weals or at a time when there was no urticaria. The difference in response to stored serum from 1984 and fresh serum taken at follow-up in 1985 is illustrated for patient 2 in Figure 1.

Positive skin tests were often evident within 30 min and were usually maximal between $1\frac{1}{2}$ and 2 h. They remained positive for an average of 8 h (range 4–13 h) in the four serum-positive patients who were followed. Ten patients who were re-injected with the same serum between $1\frac{1}{2}$ and 19 h later (mean $14\frac{3}{4}$ h) all showed the same response as that shown to the initial skin test.

TABLE 1. Clinical characteristics of 12 urticaria patients with serum-positive and serum-negative response to autologous serum

| | Serum-positive | Serum-negative |
|---|---|---|
| | $n = 7$ | $n = 5$ |
| Age (years): range | 24–78 | 36–62 |
| mean | 42 | 51 |
| Sex: M:F | 1:6 | 3·2 |
| Duration of disease: range | 4–36 months | 15 months–29 years |
| mean | 13 months | 12 years, 10 months |
| Duration of weals (h) | | |
| mean and (range): minimum | 2·4 (0 5–6) | 6 (2–10) |
| maximum | 23 (6–36) | 36 (24–48) |
| History of exacerbation with pressure | 3 | 4 |
| Personal history of atopy | 1 | 0 |
| Family history of atopy | 2 | 0 |

TABLE 2. Characteristics of skin test response in serum-positive patients

| | | | Initial assessment | | | Follow-up at one year | | |
|---|---|---|---|---|---|---|---|---|
| | | | State of urticaria when serum drawn | | | | Re-injection of serum | |
| Patient | Sex | Age | Erupting | Stable | Clear | Activity of urticaria | Original | Fresh |
| 1 | F | 31 | + | N/R | + | Almost clear<br>No treatment | − | − |
| 2 | F | 30 | N/R | + | + | Sporadic<br>anti-$H_1$ p.r.n. | + | − |
| 3 | F | 32 | + | + | N/R | Active<br>Regular anti-$H_1$ | + | + |
| 4 | M | 78 | + | + | N/R | Clear<br>No treatment | + | − |
| 5 | F | 64 | N/R | + | + | Sporadic<br>anti-$H_1$ p.r.n. | + | + |
| 6 | F | 32 | N/R | N/R | + | Clear<br>No treatment | + | − |
| 7 | F | 23 | N/R | + | N/R | Lost to follow-up | | |

+ = serum-positive skin test.
− = serum-negative skin test.
N/R = not recorded.
anti-$H_1$ = antihistamines.

Three serum-negative patients developed late weals at saline skin test sites, and two also developed weals at the site of serum injection between 8 and 24 h from the time of injection.

*Laboratory investigations*

All patients were HB$_s$Ag negative. There was some evidence of immune complex formation in both serum-positive and serum-negative groups. Three patients had elevated C1q binding but, of these, only the one patient who was serum negative also had a raised PAT. PEG was elevated in five of the six patients who were assayed, including one serum-positive patient who had raised C1q binding. ACA was normal in all patients. Two patients in the serum-positive group showed abnormalities suggesting activation of the classical complement pathway. Cryoglobulins were elevated in one patient from each group. The anti-nuclear factor was greater than 1 : 10 but less than 1 : 80 (homogeneous IgG) in two serum-negative and one serum-positive patient. Total serum IgE was above the normal range (up to 81 kU/l) in four serum-negative patients (639, 116, 143 and 165 kU/l respectively) and one serum-positive patient (529 kU/l). IgG was elevated in one serum-negative subject, IgA in two and IgM in two. IgA was also raised in one serum-positive subject and IgM in three. All other biochemical and haematological tests were normal except for the plasma viscosity which was raised in two serum-negative and one serum-positive patient.

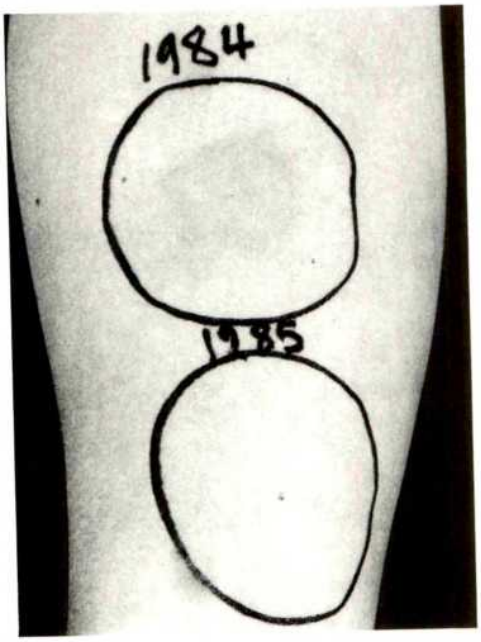

FIGURE 1. Reaction to re-injection of stored (1984) and fresh (1985) autologous serum.

### *Histology and direct immunofluorescence*

Five of the seven serum-positive and four of the five serum-negative patients showed a marked neutrophil perivascular infiltrate in the serum-injected sites (Table 3). This neutrophil reaction was much less common (two of nine) and less severe in the control subjects. Reactions to saline were dominated either by mononuclear cells or by a mixed infiltrate. Eosinophils were seen in

TABLE 3. Histological grading of infiltrate in serum and saline-injected skin (normal biopsies not tabulated)

| | | Serum | | | Saline | | |
|---|---|---|---|---|---|---|---|
| | | Neutrophil predominant | Mixed | Mononuclear predominant | Neutrophil predominant | Mixed | Mononuclear predominant |
| Serum-positive $n = 7$ | mild | — | — | — | — | 3 | 3 |
| | moderate | 2 | — | — | — | — | — |
| | marked | 5 | — | — | — | — | — |
| Serum-negative $n = 5$ | mild | — | — | — | — | 1 | 1 |
| | moderate | — | 1 | — | — | 1 | 2 |
| | marked | 4 | — | — | — | — | — |
| Controls $n = 9$ | mild | — | 2 | 1 | — | 2 | 5 |
| | moderate | 2 | — | 2 | — | — | — |
| | marked | — | — | — | — | — | — |

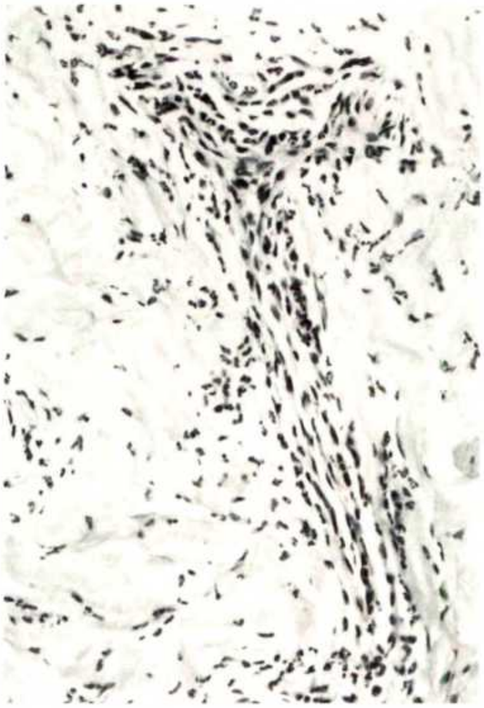

FIGURE 2. Neutrophil infiltration within and around a dermal venule in serum injected skin. (H & E; original × 213).

some serum-positive and some serum-negative patients in both serum and saline-injected skin but were rare in control subjects by comparison. The infiltrates were always perivascular, involving vessels at all levels of the dermis, but were also seen when the inflammatory changes were intense in collagen and around skin appendages. Migration of neutrophils through vessel walls was commonly observed when the infiltrate was marked (Figs 2 and 3) but leukocytoclasis, erythrocyte extravasation and fibrinoid changes in the vessel walls were not seen.

Direct immunofluorescence showed weak dermal deposition of fibrin in one serum-positive patient, weak perivascular staining with IgM in another and weak perivascular fibrin in a third, but was negative in all saline-injected biopsies and control subjects.

## DISCUSSION

Our studies show that the serum of some patients with chronic urticaria contains a mediator which will produce a weal on re-injection into their skin. The concept that the presence or absence of this mediator correlates with disease activity is strongly supported by the persistence of a positive skin test in five out of six of the patients re-tested with their stored serum after a year, particularly as two patients became serum negative after their urticaria had cleared. The two patients who continued to show a positive response with fresh serum also had continuing disease activity.

The nature of this circulating mediator remains uncertain. There was some serological evidence of immune complex formation in our patients, although in all but one the evidence was

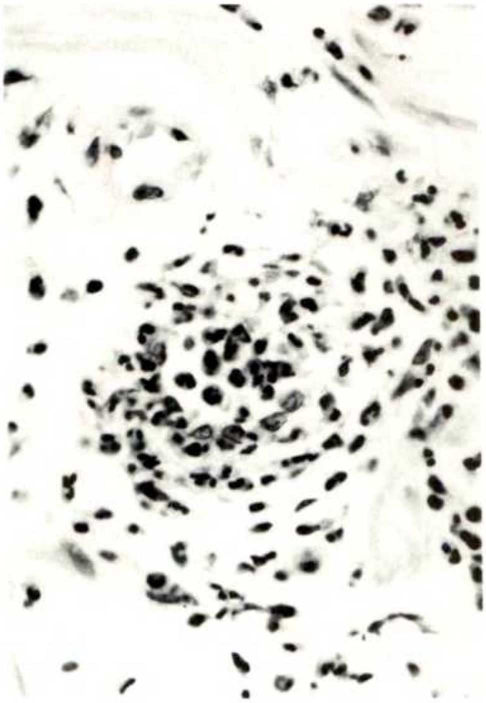

FIGURE 3. Detail of neutrophil perivascular infiltrate. (H &E; original × 465).

slight and did not correlate with skin test reactivity. Only two patients showed evidence of complement consumption. The absence of unequivocal evidence of immune complex formation may be due to insensitivity of the detection systems used, and does not provide formal evidence of their absence from the circulation. Peters and Winklemann (1985) found no evidence of complement abnormalities in a series of 10 patients with urticaria who showed the histological picture of neutrophil infiltration within and around blood vessels of spontaneous weals. They suggested that this appearance may imply the presence of physical urticaria rather than vasculitis.

Reports that symptomatic dermographism may be mediated by IgE (Aoyama, 1971; Newcomb & Nelson, 1973) suggest a possible role for an immunoglobulin-mediated response. However, as the positive skin test response is produced by serum from the same individual it is unlikely to result from IgE 'autosensitization' and subsequent mast cell degranulation. Horiko and Aoki (1984) described the successful passive transfer of IgM-mediated wealing in three patients with symptomatic dermographism and also showed that one patient reacted with a weal to his own serum. They speculated that the IgM was causing direct or antibody-mediated damage to the mast cell. However, the time course and duration of the weals was much shorter than in our series, arising in 15 min and disappearing by 30 min, in contrast to the positive responses in our patients which were usually maximal between 90 and 120 min and lasted for around 8 h.

Whilst the presence of a positive skin test provides evidence of an inflammatory serum mediator, it is too early to be sure whether it also carries prognostic significance in clinical practice. The similar incidence of neutrophil infiltration in serum-positive and negative skin tests and the relatively arbitrary definition of the criteria for a positive response emphasize that the distinction between serum-positive and serum-negative patients is not absolute. However, the longer duration of spontaneous weals, disease activity and the more frequent history of exacerbation with pressure in the serum-negative patients suggest that they may belong to a distinct clinical sub-group, which can be identified by the skin test response.

## ACKNOWLEDGMENT

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# Weathering of hair in trichothiodystrophy

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## SUMMARY

The structure of hairs from three patients with sulphur-deficient hair, trichothiodystrophy, has been studied by light, plane-polarizing and scanning electron microscopy. Severe cuticular and secondary cortical degeneration was evident along almost the entire length of the hair shaft, with cuticle loss, trichorrhexis nodosa formation and trichoschisis. The severity of these changes varied between patients. The role of the cuticle in protecting hair from environmental damage is discussed.

Trichothiodystrophy is a rare congenital disorder of hair with characteristic light and polarizing microscopic appearances and a strikingly low sulphur content (Price *et al.*, 1980). Clinically, the hair is short, fragile and sparse. Other severe congenital neuro-ectodermal changes may occur, though none is a constant finding. These include mental and physical retardation, lamellar ichthyosis, ocular dysplasia, nail dystrophy, dental caries and impaired fertility. Polarizing microscopy shows characteristic bright and dark bands along the length of the hair shaft. The sulphur content of the hair proteins is reduced to roughly 50% of normal, and this reduction affects the sulphur-rich globular matrix proteins of the cortex, as well as the cuticular A-layer and exocuticle (Gillespie & Marshall, 1983; Gummer, Dawber & Price, 1984). It is postulated that the absence of high-energy disulphide bonds in these sites is responsible for the poor mechanical properties of the hair shaft.

Normal hair fibres undergo progressive degenerative changes from root to tip due to a variety of environmental and cosmetic insults. The morphology of weathered normal hair has been previously described (Swift & Brown, 1972; Garcia *et al.*, 1978). The neatly overlapping cuticular scales of newly formed hair became irregularly raised and broken, and ultimately entirely lost, often with longitudinal fissuring of the underlying denuded cortex. Severely damaged hairs show nodes of the trichorrhexis nodosa type. These changes become progressively more severe from the root to the tip of the hair shaft, and there is frequently fraying of the tip.

The aim of this study was to investigate the type of weathering of the hair shaft occurring in trichothiodystrophy, to give us further insight into the function of the cuticle, in particular the A-layer in resisting normal, potentially damaging, physical forces.

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## METHODS

Hairs were obtained from the scalps of three affected individuals. The hairs were examined by light and plane polarizing microscopy. Hairs for scanning electron microscopy were mounted on stubs, sputter coated with gold and examined in a Philips 505 scanning electron microscope. Thirty to fifty hairs from each patient were examined by light microscopy, and six to nine hairs from each patient by scanning electron microscopy.

## RESULTS

All of the hairs examined were very short, with a maximum length of 2·2 cms.

### *Light microscopy*

The hair shafts showed irregular variations in width, and there were frequent twists about the long axis of the hair, giving rise to an appearance resembling pili torti. In places, the hair shaft was completely folded on itself. Trichorrhexis nodosa-type breaks were commonly seen, as were completely fractured hair shafts, with marked fraying of the broken ends (brush breaks). Both of these appearances were present close to the root end of the hair as well as distally. Trichoschisis breaks were also seen, though less frequently. Many of the distal tips of the hairs were severely frayed and split (trichiloptosis). Under plane polarizing microscopy, the hairs showed distinctive alternating light and dark bands. All of the hairs examined showed these features in varying degrees.

These findings agree with those of other authors (Price *et al.*, 1980).

### *Scanning electron microscopy*

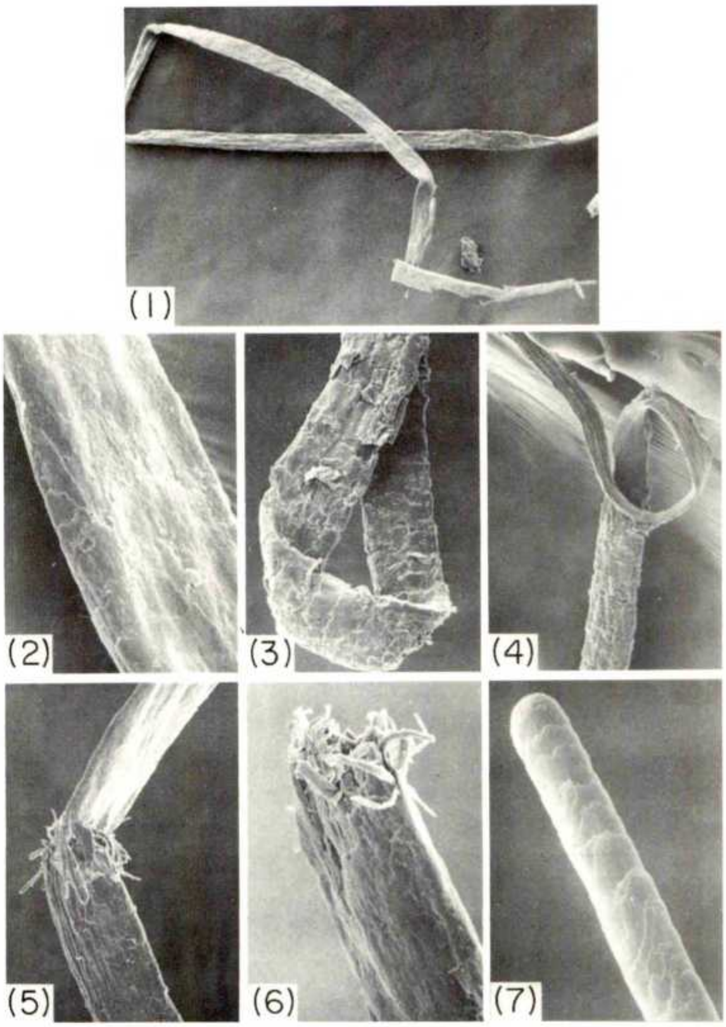
Low power views showed the hair shafts to be flattened in cross section and longitudinally ridged (Fig. 1). As with light microscopy, the shafts showed twists and folds suggesting a lack of rigidity and reminiscent of a piece of tape (Fig. 1). High magnification of the root end of the hair showed that a cuticular layer was present but incomplete. In places, the overlapping free edges of the cells were clearly visible, but had a 'tattered' appearance, with flaking of cuticle fragments. In other areas of the proximal shaft, the cuticular layer was barely discernible (Fig. 2).

In the mid portion of the shaft, there was even more marked disruption of the cuticle. Transverse splits in the cuticle cells were evident and the free margins of the cells were lifted and extensively abraded (Fig. 3).

The degree of cuticular loss was most striking close to the tip of the hair where large areas of the cortex were entirely denuded (Fig. 4). The exposed cortex showed longitudinal splitting and irregular coarse pits (Figs. 4, 5, 6).

Partial breaks of the trichorrhexis nodosa type, as well as complete brush breaks, were seen. At these sites the cuticle was extensively damaged or lost, and the cortical cells showed severe longitudinal splitting and breakage with protrusion of the fractured cortical cells from the hair shaft (Fig. 5). Similarly, at the hair tip, the cortical cells were grossly split and frayed (Fig. 6).

These findings were more striking in hairs from two of the patients; in the third, the cuticular degeneration was much less severe, and several of the hairs showed an intact cuticular layer right up to the tip (Fig. 7). Trichorrhexis nodosa breaks and obvious cuticular weathering were present in this patient, though they were less severe.



Scanning electron micrographs of hairs from patients with trichothiodystrophy. FIGURE 1. Root end of hair shaft. The flattened bore of the hair shaft and the presence of longitudinal ridges is illustrated. (× 180). FIGURE 2. Proximal portion of hair shaft showing the incomplete cuticular layer with abrasion and flaking of the cells. (× 2500). FIGURE 3. Mid portion of hair shaft in which transverse splits through the cuticle with extensive lifting and abrasion of the cuticular cells can be seen. (× 650). FIGURE 4. Distal portion of a hair shaft showing extensive loss of the cuticular layer with longitudinal ridging and splitting of the exposed cortex. (× 350). FIGURE 5. Trichorrhexis: The partial transverse break shows splaying outwards of the cortical cells and the almost total absence of the cuticular layer. Note the coarse depressions of pits in the exposed cortex (× 825). FIGURE 6. Tip of hair shaft showing the absence of the cuticular layer and the frayed appearance of the cortex (× 2200). FIGURE 7. Hair tip from the least severely affected patient showing the normal appearing cuticular cells (× 1650).

## DISCUSSION

These findings confirm previous reports suggesting that, unlike normal hair, trichothiodystrophy hair lacks mechanical strength and resilience and behaves in a similar fashion to a floppy ribbon, being flattened in cross section, and showing folds and pseudo-twists (Price *et al.*, 1980).

In addition, we have described in more detail the degenerative changes seen in trichothiodystrophy. Affected hair shows an advanced degree of degeneration or weathering, with lifting, abrasion and loss of cuticle cells, brush breaks, pitting and longitudinal splitting of the exposed cortex, and fractures of the trichorrhexis nodosa and trichoschisis types. These changes were observed in both the proximal and the distal portions of the hair shaft. Similar abrasion and loss of cuticle cells, and even trichorrhexis nodosa breaks, are seen in normal hair, but to a much lesser extent and usually towards the tip of relatively long hairs. As a rule, the finding of proximal trichorrhexis nodosa implies an intrinsic hair shaft defect.

In the three patients studied, the severity of damage to the hair showed considerable variation, one of the patients being only mildly affected compared to the other two; indeed, many of the hair tips in this patient were well preserved (Fig. 7). It is unknown whether this variation in severity reflects the differing degrees of trauma to which the hair has been subjected, or whether there is variation in the severity of the underlying biochemical defects. The latter would appear to be the more likely.

Trichothiodystrophy is unique among congenital hair abnormalities in that the biochemical basis of the hair abnormality has been partially elucidated. The sulphur and cystine content of the hair are both reduced to approximately 50% of their normal values, the reduction occurring in the ultra-high sulphur proteins of the cortex. These comprise the globular matrix proteins of the cortex in which bundles of microfibrils are embedded (Price *et al.*, 1980; Gillespie & Marshall, 1983). During normal keratinization, oxidation of thiol groups in cysteine moieties produces high-energy disulphide cross-links in the matrix proteins which contribute to the great mechanical strength of hair. It is reasonable to postulate that the lack of disulphide cross links accounts for the 'floppiness' and fragility of the hair shaft in trichothiodystrophy.

In addition to the abnormalities in the cortical proteins, it has been demonstrated by sensitive histochemical techniques that there is reduced uptake of silver stain (a cystine-positive stain) in the cuticle, affecting both the A-layer and the exocuticle. The cuticle cell has a lamellar structure, consisting of A-layer, exocuticle, endocuticle, and inner layer (Gummer *et al.*, 1984). The A-layer is the outermost layer and lies immediately subjacent to the cell membrane complex, and the exocuticle lies deep to the A-layer. In normal fully keratinized hair both the A-layer and the exocuticle are rich in cystine and contain many high-energy disulphide bonds, rendering the cuticle an extremely resilient and protective surface layer. This dense high sulphur protein layer also acts as an 'exoskeleton' in normal hair, maintaining hair shape and resisting potential twisting and bending damage; in trichothiodystrophy, the trichoschisis and trichorrhexis nodosa are due to such deformations fissuring the cuticle and exposing the underlying cortex.

The gross abnormalities seen in trichothiodystrophic hair are attributable primarily to acquired degenerative changes (weathering) rather than inherent morphological abnormalities of the cuticle, well-formed cuticular cells being present at the proximal end (Fig. 2), and in a few short hairs being well preserved even at the hair tip (Fig. 7). This evidently suggests that an intact cuticle layer is formed by the hair follicle, but because of the deficiency of high-sulphur proteins, particularly in the cuticular A-layer (Gummer *et al.*, 1984), the hair is unable to withstand normal environmental physical and chemical weathering.

The important protective role of the cuticle in normal hair has been suggested by a number of authors (Swift & Brown, 1972; Papa, Mills & Hanshaw, 1972; Orfanos & Ruska, 1968; Wolfram & Lindemann, 1971). Scanning electron microscopic studies of the effects of trauma (e.g. saline soaks, shampooing and exposure to UV light) on clinically normal hair shafts have shown stripping of the cuticular layer, and Papa, Mills and Hanshaw (1972) believe that this is the earliest observed precursor to clinically evident trichorrhexis nodosa fractures. Robbins and Kelly (1969) have demonstrated a reduction in cystine content in bleached and permanent-waved hair, though they do not report whether this reduction affects the cortex or cuticle or both.

Clinically, trichothiodystrophy hair is sparse, fragile and fails to attain a normal length. These clinical features may be attributed to the failure to incorporate cystine normally into the keratinizing shaft, and the consequently poor mechanical properties of the hair. In particular, the advanced degree of cuticular weathering can be related to the defective formation of the outer layers of the cuticle, especially the A-layer.

It is unknown why the follicle fails to produce normal cystine-containing proteins, as there is no evidence for a generalized defect in cystine transport, uptake or utilization. Gummer and Dawber (1985) have recently described the morphological abnormalities in the anagen hair follicle in trichothiodystrophy. The lower portion of the hair follicle, both below and within the 'zone of keratinization', shows gross spatial distortion of all cell layers. This is accompanied by a reduction of protein deposition, as well as abnormal orientation of the proteins, in both the cortex and the cuticle. The cortical fibrillar proteins fail to align themselves parallel to the long axis of the hair shaft, while the cuticular sulphur-rich proteins are deposited randomly throughout the cuticle cell, instead of aggregating close to the outer cell membrane. These authors suggest that keratinization is attempted but fails to be completed; consequently, the dynamic forces resulting from cell division and growth of the hair bulb and outer root sheath produce distortion of the partially-keratinized follicle and hair shaft. The component of keratinization which is lacking in trichothiodystrophy is unknown, but it has been postulated that there is an alteration in a regulatory gene (Gillespie & Marshall, 1983).

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# Cutaneous schistosomiasis in Nigeria. An update

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## SUMMARY

Two patients with schistosomiasis of the skin were seen at the Ahmadu Bello University Teaching Hospital, Kaduna, Northern Nigeria, between June 1978 and December 1980. The first case was not suspected on clinical grounds, but was diagnosed only after terminally-spined ova of *Schistosoma haematobium* were found in histological sections of the skin lesions. Both patients were successfully treated with niridazole (Ambilhar®). These bring to eight the total number of cases of cutaneous schistosomiasis recorded in Nigeria in the past 40 years, four of which were in indigenous Nigerians. It seems that the high endemicity of the disease confers some degree of natural immunity to the indigenous population, making ectopic forms rare. Clinicians should be aware of the existence of cutaneous forms of the disease and of their excellent response to specific chemotherapy.

Schistosomiasis, a parasitic disease of the genito-urinary and intestinal systems, remains endemic in many parts of the tropics and subtropics. In Nigeria, as in other parts of Africa, the exploitation of water resources in the form of man-made lakes for agricultural expansion has created new foci for the transmission of the disease.

The low standard of personal hygiene as well as inadequate health education programmes favour the spread of the disease not only in rural communities but also in urban areas like Kaduna and its environs. Despite the efforts of the World Health Organization (W.H.O.) towards its control and ultimate eradication, the infection seems to be continuing to spread. The symptoms and signs of the genito-urinary and intestinal forms of the disease are so well known that the diagnosis is readily made. Ectopic forms of the disease are not easily diagnosed on clinical grounds without histological examination, because they have no characteristic features.

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The first of the two cases was presented at the Annual Meeting of the Association of Physicians of Nigeria, Benin City, Nigeria, in December 1979.

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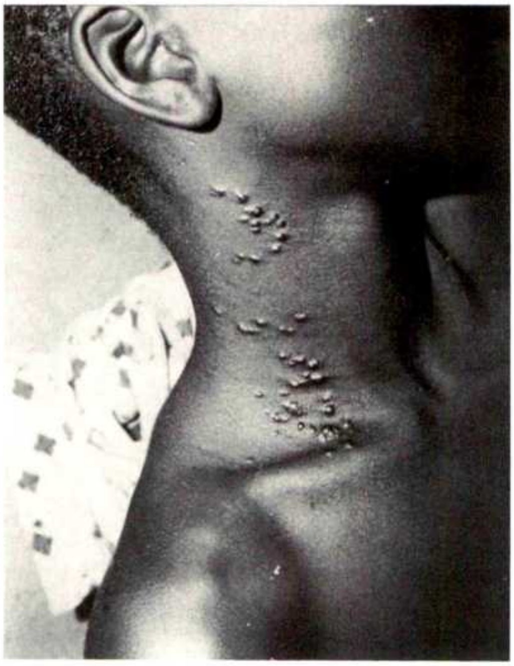

FIGURE 1. Case 1: clusters of hyperpigmented lichenoid papules on the right side of the neck and right supraclavicular region.

## CASE REPORTS

### *Case 1*

A 12-year-old boy was first seen at our clinic in June 1978. He gave a one-month history of an itchy papular rash on the right side of his neck. This was treated with topical cream at the school clinic with no response. He was otherwise well and no other member of his family was affected. Examination revealed a healthy looking boy with clusters of hyperpigmented papules on the right side of the neck and right supraclavicular area (Fig. 1). The diagnosis was not clear at that stage, and 4 days later a biopsy was taken from the lesion. At follow-up 3 weeks later, he had developed haematuria without diarrhoea or blood in his stools. Microscopy of a terminal drop of urine showed numerous red blood cells, several ova of *Schistosoma haematobium*, a few epithelial cells and pus cells. He was admitted to hospital for fuller investigations and treatment.

Bacteriological culture of urine, and stool examination were negative. A rectal snip contained ova of *S. mansoni*. His haemoglobin (Hb) was 12·0g/dl., PCV 36·0, MCHC 34, total WBC $4{\cdot}6 \times 10^9$/l with 62% neutrophils, 36% lymphocytes and 2% eosinophils. The Hb electrophoretic pattern was AA, and erythrocyte sedimentation rate was 14 mm/hour (Westergren). Liver function tests, urea and electrolytes were normal. Serum IgM was 217 IU/ml (normal up to 760 IU/ml). There was no calcification on plain X-ray of the abdomen, and intravenous pyelography showed normal kidneys and ureters. There was post-micturition residual urine in the bladder which showed thickened mucosal folds. The pathologist's report of the skin biopsy read: 'a beautiful case of schistosomiasis of the skin' with 'three or four schistosome ova with a characteristic epithelioid cell reaction around them leading to pseudotubercle formation'

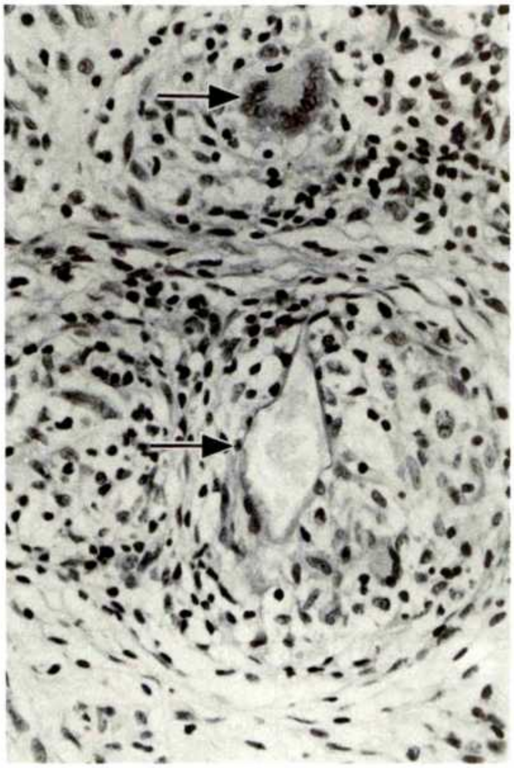

FIGURE 2. Case 1: histological section from skin biopsy. Dermal schistosomal granuloma showing *S. haematobium* ovum (lower arrow) with a characteristic terminal spine in the centre of a pallisading granuloma, and a giant cell at the (upper arrow).

(Fig. 2), 'keratotic plugging of two hair follicles and an infiltrate of lymphocytes and eosinophils around them'.

In August 1978, treatment was started with niridazole (Ambilhar®) 250 mg three times daily for 7 days. Haematuria stopped 3 days after initiating therapy and there were no side effects. The papular lesions were regressing prior to discharge from hospital at the end of this course of treatment. At follow-up 11 days later, he was well and the skin papules were disappearing. Urine microscopy showed five red blood cells per field. At the last visit at the end of September, 1978, he had no urinary symptoms, no haematuria, and the papules had completely disappeared, leaving areas of post-inflammatory hyperpigmentation.

### *Case 2*

A 10-year-old boy was seen at our clinic and admitted to hospital in December 1980 with a history of an itchy skin eruption on the anterior aspect of the right chest wall first noticed in September of that year. The lesions were increasing in number and size. Early the following month he started having haematuria which was initially terminal but later occurred throughout micturition. He denied any drug therapy prior to the onset of the illness and there were no other symptoms. He had been swimming in a pond near his home in Ungwar Rimi village, Kaduna. He was the only one in a family of eight children who had done this, and none of the others was

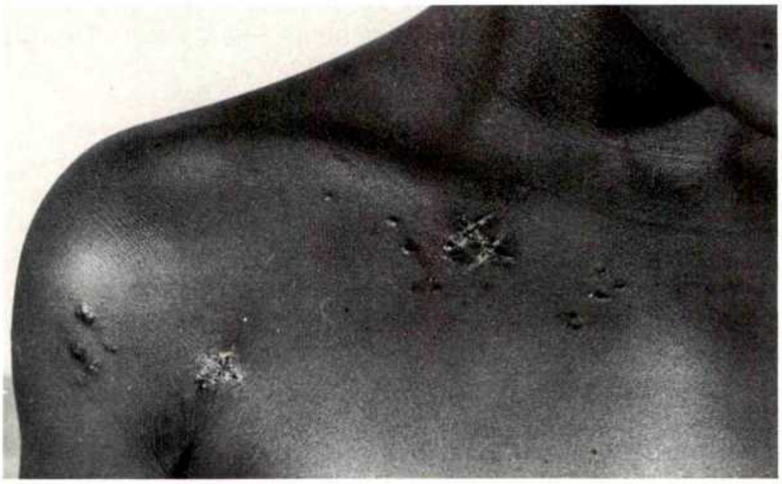

FIGURE 3. Case 2: multiple lichenoid papules on the right side of anterior chest wall below the clavicle, and on the anterior aspect of the right shoulder. Also two skin biopsy sites: below the right clavicle and above the right anterior axillary fold.

affected. He did not know of any other children in the village who had a similar illness. On examination, he was a healthy looking boy, afebrile, and not clinically anaemic. He had multiple hyperpigmented lichenoid papules distributed over the anterior part of the right chest wall below the clavicle, the anterior and posterior parts of the right shoulder (Fig. 3), and below the right nipple. There was a palpable solitary, mobile and non-tender right axillary lymph node, 1·0 × 0·5 cm. Other systems appeared normal.

On the basis of our experience with Case 1, a clinical diagnosis of schistosomiasis of the urinary bladder with cutaneous spread was made. Examination of a terminal drop of the

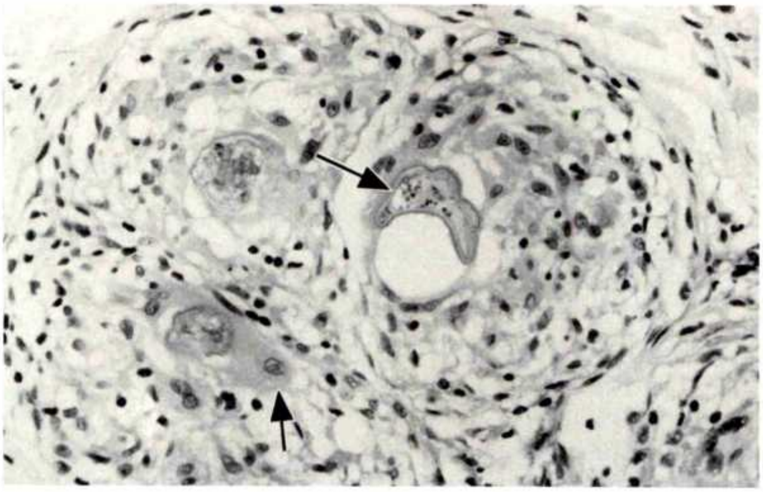

FIGURE 4. Case 2: histological section from skin biopsy. Dermal granuloma showing remnants of schistosome ova with thick cuticle (upper arrow), and intact ovum (lower arrow) with terminal spine.

patient's urine showed numerous ova of *Schistosoma haematobium*, numerous red blood cells, and pus cells. Fresh stool examination and rectal snip were negative. His Hb was 11·7 g/dl, PCV 38·5, MCHC 31, total WBC 8·3 × $10^9$/L with 13% neutrophils, 46% lymphocytes, 1% monocytes and 40% eosinophils. A blood film showed anisocytosis, macrocytosis and polychromasia. Liver function tests, serum urea and electrolytes were normal. Scalpel skin biopsy of the papules was taken from two different sites (Fig. 3), and on histology there was a granuloma with the remains of schistosome ova (Fig. 4). He was treated with niridazole (Ambilhar®) 200 mg three times daily for 7 days, with no side effects, and discharged on completion of this treatment. At out-patient follow-up a week later, there was no haematuria and the skin papules were disappearing. At his last visit in January 1981, the papules had almost completely disappeared, and he had no urinary symptoms.

## DISCUSSION

The genito-urinary and intestinal forms of schistosomiasis are very common, but ectopic forms of the disease are rare among indigenous Nigerians. Prior to the present report, in the past 40 years only six cases of ectopic cutaneous schistosomiasis have been reported from Nigeria. The first four affected British troops training for jungle warfare during the second world war (Black, 1945).

Adeyemi-Doro, Osoba and Junaid (1979) reported a case of perigenital cutaneous schistosomiasis, affecting a 10-year-old Nigerian, boy and Jacyk, Lawande & Tulpule (1980) reported the 'unusual presentation of extragenital cutaneous schistosomiasis mansoni', involving the forehead and right temple of a 13-year-old Nigerian boy. The two cases presented here bring to four the cases of ectopic cutaneous schistosomiasis so far documented among indigenous Nigerians. Ectopic cutaneous schistosomiasis is, therefore, rare among Nigerians. The condition has been reported more frequently in Europeans visiting the endemic areas for a short time (Black, 1945; Findlay & Whiting, 1971; Saxe & Gordon, 1975; Wood, Srolovitz & Schetman, 1976). High endemicity of schistosomiasis in Nigeria probably accounts for the infrequent occurrence of ectopic cutaneous forms in the indigenous population by enabling a more stable host-parasite relationship to develop. Infections, therefore, conform better to type, and ectopic manifestations become rare (Findlay & Whiting, 1971; Abdel-Aziz, 1976). In a histopathological study covering a period of 5 years, only seven cutaneous lesions of schistosomiasis were found in a total of 1397 skin biopsies (Abdel-Aziz, 1976). In the Ivory Coast, a country with endemic schistosomiasis where 10 per 100 of the population suffer from bilharziasis, only 14 cases with cutaneous lesions were diagnosed by micropathology, between 1970 and 1980 (Colin *et al.*, 1980). Haber and Cowley (1957) documented a case of cutaneous schistosomiasis in a Ghanaian student in London, while MacDonald and Morrison (1976) reported another case affecting a woman from Sierra Leone. Both African immigrants had acquired their infection in their home countries, and had been inadequately treated.

Natural immunity is believed to be dependent on racial and genetic factors (Ramos & Torres, 1975). Short-term visitors to endemic areas should avoid swimming in potentially infested lakes. Clinicians should be aware of the existence of the ectopic forms of schistosomiasis and of its excellent response to specific chemotherapy.

## ACKNOWLEDGMENTS

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# Precise dermal damage with an infrared coagulator

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## SUMMARY

Using an infrared coagulator, we have shown, in pigskin, a direct relationship between depth of injury and the duration of infra-red pulses, which was highly reproducible at a particular site. This has been related to the clinical results achieved in one patient whose three tattoos were treated with different pulse times.

Port wine stains and tattoos are treated with a wide variety of physical modalities. Recent interest in laser therapy stems to a large degree from the accurate and predictable nature of the tissue destruction using these instruments. The argon light is absorbed preferentially by blood vessels, but conduction of heat to surrounding structures and secondary epidermal changes result in a non-specific wound. The tunable dye laser causes less epidermal damage; however, the clinical results using the $CO_2$ laser (Bailin, 1983) which causes epidermal vaporization, are encouraging enough to cast doubt on the idea that specific absorption of energy by vessels is important or even necessary to ameliorate the naevus flammeus.

An infrared coagulator emits non-coherent radiation which produces non-specific thermal effects. It has previously been employed to treat tattoos (Colver *et al.*, 1985), and vascular naevi (Schmoll, 1981; Colver *et al.*, 1984; Krumrey, 1984). We have found that the amount of skin damage can be carefully controlled and that healing produces aesthetically good scars.

In the present study, using pig skin, we have investigated the relationship between the duration of each pulse of radiation (using pulses of up to 1·25 s) and the depth of injury. The depth of connective tissue damage was visualized histologically using an elastic fibre stain. These results were compared with clinical results in a patient, using pulses of up to 1·5 s.

## METHODS

### *Infrared coagulator*

An infrared coagulator (Model IRK 151, MBB–AT Munchen) with a 15-volt, 120-watt

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tungsten halogen bulb was used. The coagulator consists of a hand piece which is connected to the mains electricity supply via a transformer. The bulb emits a spectral band of 400–2700 nm and emission is maximal at 900–960 nm. The radiation is reflected down a rigid quartz glass light guide by a gold-plated reflector. The light guide had a 6 mm sapphire cap which is applied to the skin. By pre-setting an electronic timer, the operator is able to vary the duration of each pulse between 0 and 1·5 s.

### *Animal skin*

Large White pigs weighing approximately 20 kg were used. Anaesthesia was induced with ketamine hydrochloride and maintained with oxygen, nitrous oxide and halothane.

Two areas of flank skin on one pig and the flank plus two areas on the chest wall of the second pig were used. Each area measured 2·5 × 15 cm and was divided into six equal squares of 2·5 × 2·5 cm. Each square received a single pulse of 0·500, 0·625, 0·750, 0·875, 1·000 or 1·125 s. Thus, each of five separate sets of squares was subjected to a series of pulses ranging from 0·5 to 1·125 s.

Biopsies were taken after 20 min, fixed in 10% buffered formalin for 24 h and embedded in paraffin wax. 5-$\mu$m sections from each biopsy were stained with haematoxylin and eosin, and with Verhoeff's elastic/van Gieson technique (Drury & Wallington, 1980).

Biopsies were examined by light microscopy without knowledge of the pulse times. Depth of injury was measured with an eye-piece micrometer. The stains differentiate between altered

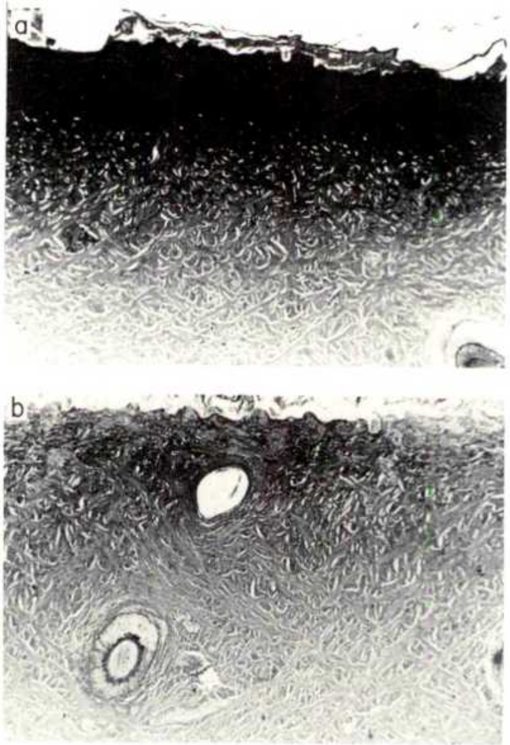

FIGURE 1. Pig skin after infra-red treatment. (a) 1·0 s pulses and (b) 0·875 s pulses. (Verhoeff's elastic/van Gieson technique; original × 35).

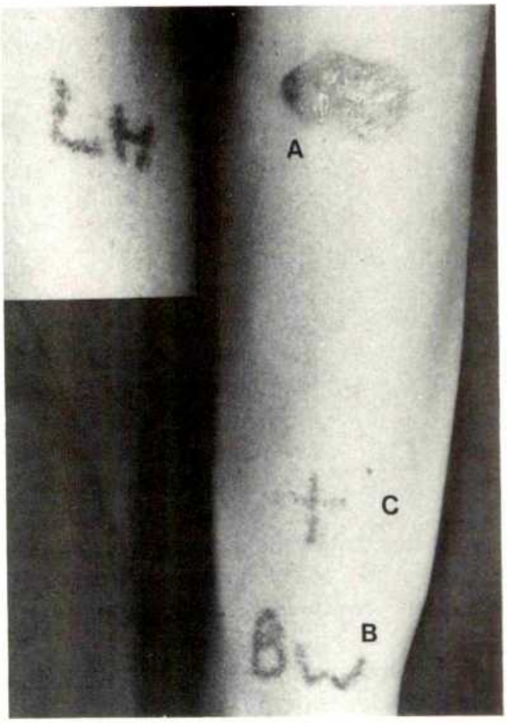

FIGURE 2. Patient's forearm showing tattoo 'A' before (inset) and 2 months after treatment, and tattoos 'B' and 'C' before treatment.

collagen which retains the black elastic fibre stain, and viable collagen which stains pink. There was an intermediate zone staining brown and purple which may represent injured but viable collagen. There was a distinct cut-off between black and purple collagen, and the depth of injury was assessed by measuring from the dermo-epidermal junction to this point. The microscopic appearance is shown in Figure 1. When the epidermis was missing, measurement was made from the top of the denuded surface.

### *Human skin*

A 24-year-old Caucasian female came to the clinic for removal of three decorative tattoos on the dorsal aspect of her left forearm (Fig. 2). They were treated with the infrared coagulator under local anaesthesia. During treatment the coagulator tip was moved from spot to spot with slight overlap until the area of the tattoo had been covered. One pulse of radiation was given to each spot. The proximal tattoo 'A' was treated with pulses of 1·5 s. Two months later, the distal tattoo 'B' was treated with pulses of 1·25 s and the middle one 'C' with pulses of 1·125 s. The subject was taking part in our original tattoo series (Colver *et al.*, 1985) in which an ice cube was applied to the lesion for 5 s before each pulse in an attempt to minimize epidermal damage.

## RESULTS

In the pigs, the depth of damage at the centre of the lesions was slightly greater than at the periphery. Table 1 gives the depth of injury in the centre of the lesions and 0·5 mm from the

TABLE 1. Pig skin—mean depth of injury in the centre and 0·5 mm from the edge of flank and chest lesions produced by different pulse durations

| | Depth of injury (mm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pig A | | | | Pig B | | | | | |
| | Flank | | Flank | | Flank | | Chest | | Chest | |
| Pulses (s) | Centre | Edge | Centre | Edge | Centre | Edge | Centre | Edge | Centre | Edge |
| 0·625 | 0 | 0 | 0 | 0 | 0 | 0 | 0·17 | 0·14 | 0·26 | 0·18 |
| 0·75 | 0·27 | 0·27 | 0·31 | 0·27 | 0·23 | 0·2 | 0·42 | 0·38 | 0·47 | 0·38 |
| 0·875 | 0·36 | 0·31 | 0·51 | 0·36 | 0·66 | 0·45 | 0·51 | 0·45 | 0·65 | 0·52 |
| 1·0 | 0·72 | 0·50 | 0·72 | 0·62 | 0·78 | 0·6 | 0·78 | 0·68 | 0·72 | 0·52 |
| 1·125 | 0·81 | 0·72 | 0·87 | 0·76 | 0·96 | 0·9 | 0·92 | 0·78 | 0·93 | 0·75 |

periphery for pulses of different lengths. On the assumption that flank skin differs from that over the chest wall, the results have been analysed separately. Least squares regression analysis was used to examine the relationship between pulse duration and depth of injury. Figure 3 shows this relationship, which appears to be linear, with the lines of best fit. For each location pulse duration accounted for a large amount of the variation in depth of injury ($R^2 > 0·90$). There was a significant difference between the slopes of the two lines ($P < 0·001$; Student's *t*-test).

In the patient, tattoo 'A', treated with 1·5-s pulses, developed a thick crust during the first 10 days which persisted for one month; purulent tissue was evident during that time. Tattoo 'B'

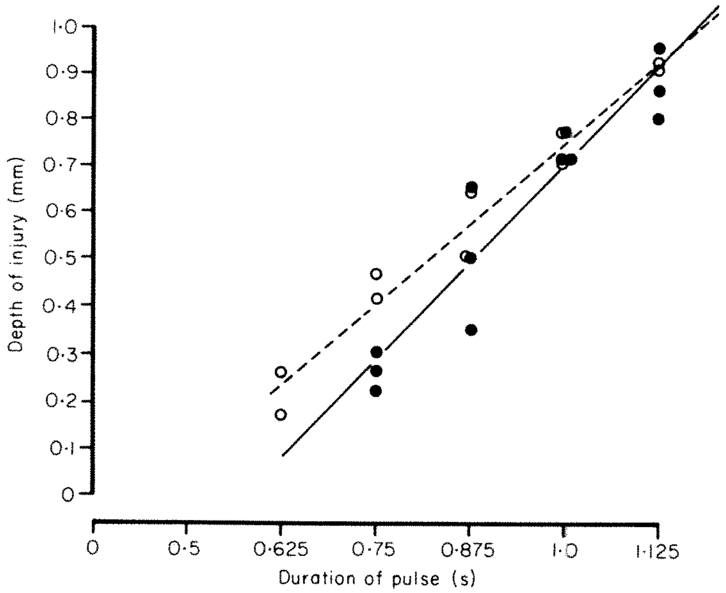

FIGURE 3. Relationship between depth of injury and pulse duration in pig skin. (●——●) flank, $y = -0·945 + 1·648x$; (○- - -○) chest, $y = -0·624 + 1·38x$.

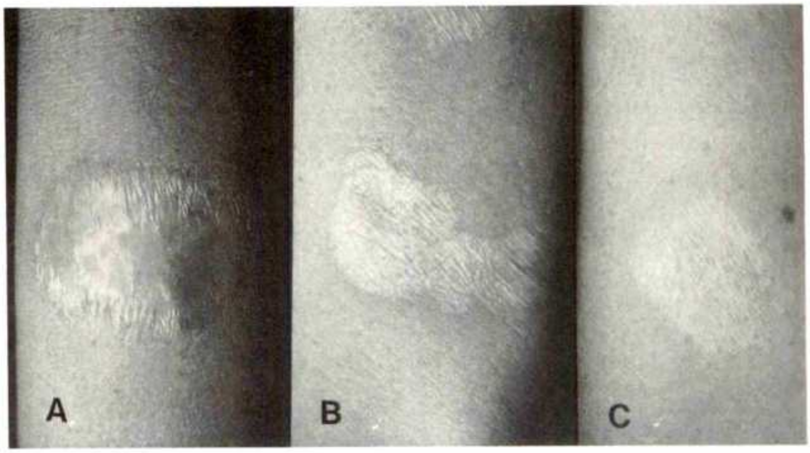

FIGURE 4. Close up views of treated tattoos 'A', 'B' and 'C' 7 months after treatment of 'B' and 'C'.

developed a crust within 6 days which remained for 3 weeks, and tattoo 'C' formed a crust within 3 days, which cleared by 10 days. There was no pain during the healing phase after the local anaesthetic had ceased to act. The wounds of 'B' and 'C' remained dry and required only a protective elastoplast. Figure 4 shows close-up views of each treated area 7 months after treatment of 'B' and 'C'. The superior results associated with shorter pulses can be seen. The patient regarded the results of removal of tattoo 'C' as 'ideal', 'B' as 'good' and 'A' as 'worthwhile'.

## DISCUSSION

The infrared coagulator has been used to treat haemorrhoids (Templeton *et al.*, 1983), benign cervical conditions (Massinger & Nath, 1979) and vascular naevi (Schmoll, 1981; Colver *et al.*, 1984; Muhlbauer, Nath & Kreitmair, 1976). In a tattoo study, those lesions treated with 1·25 s or less had a satisfactory outcome whereas those treated with pulses longer than 1·25 s developed less satisfactory scars. The benefit of pre-cooling with an ice cube has yet to be determined. (Colver *et al.*, 1985). The sapphire tip has a high thermal conductivity and appears to give better results than a teflon tip.

Several methods of treating flat cutaneous lesions have produced inconsistent results because the depth of injury varies over the field of treatment, e.g. abrasive technique and electro cautery. When the coagulator is triggered the bulb emits visible and infrared radiation. The total energy output per second increases and the spectrum changes as the filament gets hotter. It is thus very difficult to measure the energy received by the skin. However, the present study shows that the coagulator produces consistent results in pig flank and chest skin and that the depth of injury is directly related to the pulse duration. The statistical analysis does not permit extrapolation outside the pulse times used; although there is a straight line relationship between depth of injury and pulse duration from 0·625 to 1·125 s, it is likely that there would be flattening of the curve with longer pulses. In the pigs, the two skin areas tested showed different thresholds of injury and different dose responses. In humans, it is likely that several factors will affect response, including body site, pigmentation and skin type.

The infrared coagulator shares some properties with argon lasers. There is good penetration of the radiant energy and, when pulsed, there is a predictable degree of thermal damage. The $CO_2$ laser has the advantage over both these instruments of minimal heat conduction to surrounding tissues. A drawback of the coagulator in its present form is that slightly greater damage occurs in the centre of the 6 mm circle than at the periphery (Table 1). This occurs because of poor heat dissipation in the centre. We hope to overcome this by modifying the structure of the quartz light guide. At present it is necessary to overlap the treatment circles by about 1 mm to maintain an even pattern of injury over the area. As a method of assessing damage to dermal structures the stain used in this study is insensitive because it shows only destruction of collagen. When treating vascular naevi, pulse durations of 0·4 s have produced lightening of lesions over a few months but the same pulse produces no changes in collagen detectable histologically. More subtle tests are required to demonstrate damage to blood vessels.

## ACKNOWLEDGMENTS

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# Site variation in anthralin inflammation on forearm skin

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## SUMMARY

The effect of application site on anthralin inflammation was measured at 10 clinically normal volar skin sites on each forearm of 31 subjects as the increase in skin thickness at 48 h using Harpenden calipers. Pre-treatment and increase in skin thickness were significantly related to application site. Pre-treatment thickness increased distally and laterally by an amount depending on sex, and to a lesser extent other factors, whilst the increase in skin thickness values was greater proximally, laterally and on the right arm but was not affected by age, sex or skin type. When increases in skin thickness values were adjusted for pre-treatment thickness, the difference between proximal and distal sites was lost but there was still a significant difference between the medial and lateral aspect and the right and left arms. The absolute differences were small but demonstrate the need to use symmetrical forearm skin sites and randomized treatment sides when comparing the effects of topical agents on anthralin inflammation.

Forearm skin is the most suitable site for the assessment of an inflammatory response by measuring the change in skin thickness (Cook & Shuster, 1980; Moss, Friedmann & Shuster, 1981; Lawrence, Howel & Shuster, 1984; Lawrence & Shuster, 1985). In the course of recent studies of factors affecting anthralin inflammation (Finnen, Lawrence & Shuster 1984; Lawrence, Finnen & Shuster, 1984; Lawrence & Shuster, 1984) it appeared that the location of the application site on the forearm might influence the degree of response. We have therefore measured the variation in skin thickness at different sites on the forearm, and the influence of the application site on the inflammatory response of forearm skin to anthralin.

## METHOD

Thirty-one patients with psoriasis were studied, mean age 42 (range 17–68) years, evenly distributed with respect to age (15 patients $<40$ years, 16 patients $>40$ years), sex (16 male, 15 female) and skin type (18 type I or II, 13 type III or IV). Patients were chosen by their

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willingness to take part in the study. Thirty of them were right handed. The numbers of patients with each combination of age group, sex and skin type were as follows:

| | | Males | | Females | |
|---|---|---|---|---|---|
| | | Age | | Age | |
| | | <40 | >40 | <40 | >40 |
| Skin type | I or II | 3 | 5 | 7 | 3 |
| | III or IV | 3 | 5 | 2 | 3 |

Clinically normal volar forearm skin between a line 4 cm below the elbow crease and 4 cm above the wrist crease was studied. Ten sites on each arm were identified by dividing the area transversely into five equal strips and by a central line running from the middle of the elbow to the middle of the wrist crease. On each patient, measurements were made at 20 sites which were located by three parameters: along (five positions from elbow to wrist), across (lateral or medial) and side (the right or left arm). Anthralin inflammation was produced at each site by pipetting anthralin 100 $\mu$g dissolved in 10 $\mu$l of chloroform into a plastic well 15 mm in diameter and allowing the chloroform to evaporate off. Sites were left uncovered and unwashed for 6 h. Inflammation was measured with Harpenden skin calipers with one spring removed (Lawrence & Shuster, 1985) immediately before and 48 h after anthralin application. Three replicate readings were taken on each occasion and the results averaged. Inflammatory oedema was calculated from the difference between the mean reading before and after treatment. Analysis of variance for repeated measurements (Winer, 1971) was used to test for significant differences between sites before and after treatment allowing for possible variations between subjects related to age, sex and skin type. The analysis was performed using the BMDP statistical package (Dixon, 1981) in which the Greenhouse-Geisser correction (Greenhouse & Geisser, 1959) is applied to significance levels, and terms in the model are tested for significance by removing them singly, with replacement, from the full model. Any differences between sites or patients referred to in the results or discussion were statistically significant at the 5% level for main effects or the 1% level for interactions.

## RESULTS

### *Pre-treatment skin thickness*

The mean pre-treatment skin thickness at the different sites varied from 2·15 mm to 2·7 mm, (Fig. 1a) with higher values occurring at distal and right lateral sites. The mean thickness was greater in men than in women and the magnitude of the difference between elbow and wrist was sex related; the mean values in the men were 2·4 mm and 2·9 mm and in the women 1·95 mm and 2·15 mm respectively. Details of the statistical analysis are given in Table 1a.

### *Increase in skin thickness*

The mean increase in skin thickness at the different sites varied from 0·35 mm to 0·7 mm (Fig. 1b). There was a mean difference of 0·2 mm between wrist and elbow and the increases were slightly larger on the right arm and on lateral sites. These values did not vary significantly with age, sex or skin type. Details of the statistical analysis are given in Table 1.

There was some tendency for large increases in skin thickness values to occur at sites with a low pre-treatment skin thickness and vice versa (Fig. 2), the relationship being less pronounced in some subjects. Therefore, an analysis of covariance was used to compare increase in thickness,

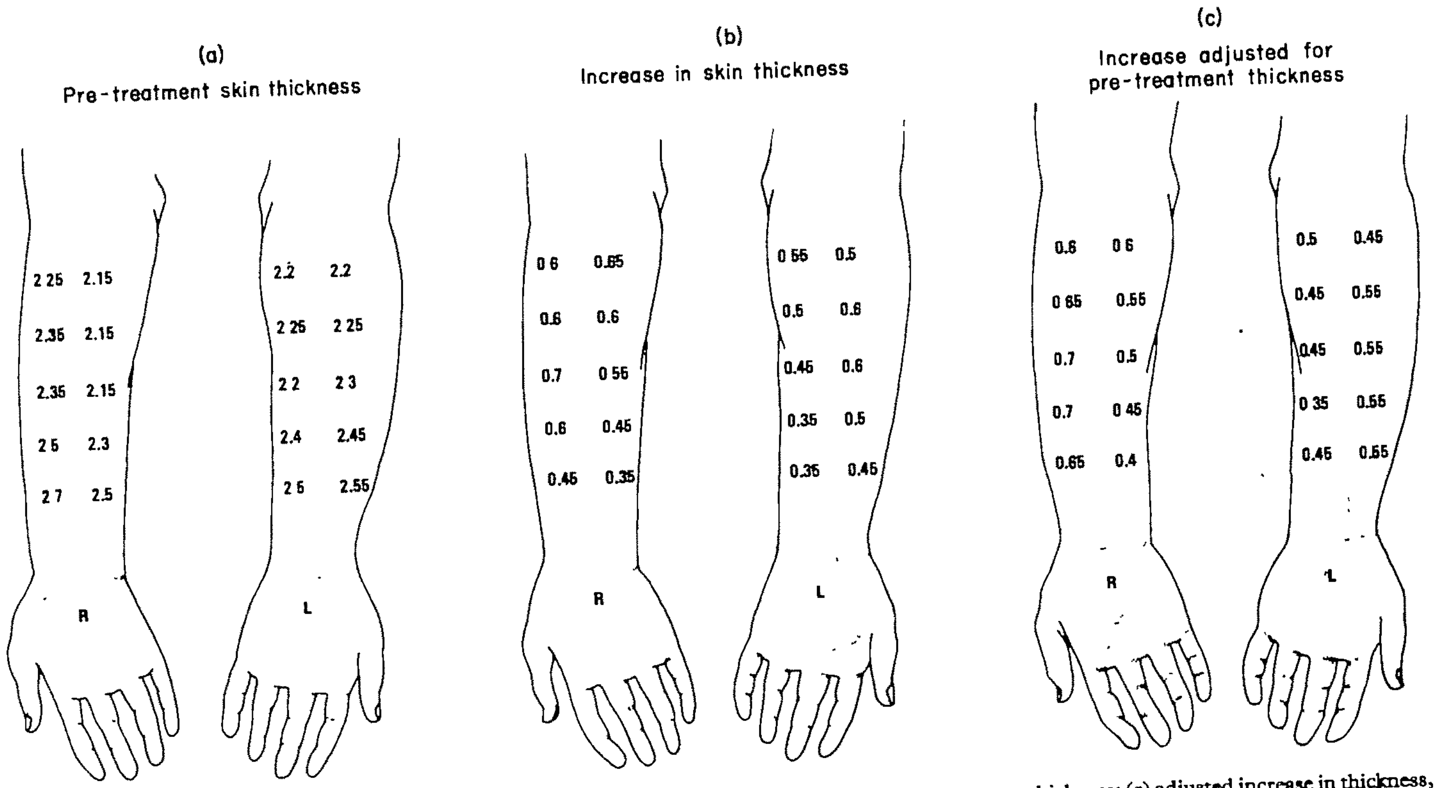

FIGURE 1. Mean skin fold thickness (mm) at 20 sites on the forearm: (a) pre-treatment thickness; (b) increase in thickness; (c) adjusted increase in thickness, assuming an original thickness of 2·3 mm.

TABLE 1. Terms found to be significant in statistical analysis

| | Term | *F* statistic | Degrees of freedom | *P** |
|---|---|---|---|---|
| (a) Pre-treatment thickness | | | | |
| | All sites | 19·65 | 19,437 | <0·0001 |
| | Along arm | 51·75 | 4,92 | <0·0001 |
| | Across arm | 18·29 | 1,23 | 0·0003 |
| | Side × across arm | 13·71 | 1,23 | 0·0012 |
| | Sex | 44·12 | 1,23 | <0·0001 |
| | Along arm × sex | 8·10 | 4,92 | 0·0009 |
| (b) Increase in thickness | | | | |
| | All sites | 6·52 | 19,437 | <0·0001 |
| | Along arm | 21·45 | 4,92 | <0·0001 |
| | Across arm | 25·99 | 1,23 | <0·0001 |
| | Side | 5·87 | 1,23 | 0·0237 |
| | Along × across arm | 4·26 | 4,92 | 0·0010 |
| (c) Increase adjusted for pre-treatment thickness | | | | |
| | All sites | 5·58 | 19,436 | <0·0001 |
| | Side | 7·91 | 1,22 | 0·0101 |
| | Across arm | 20·52 | 1,22 | 0·0002 |
| | Along × across arm | 6·60 | 4,91 | 0·0003 |

* *P* values using Greenhouse-Geisser correction.

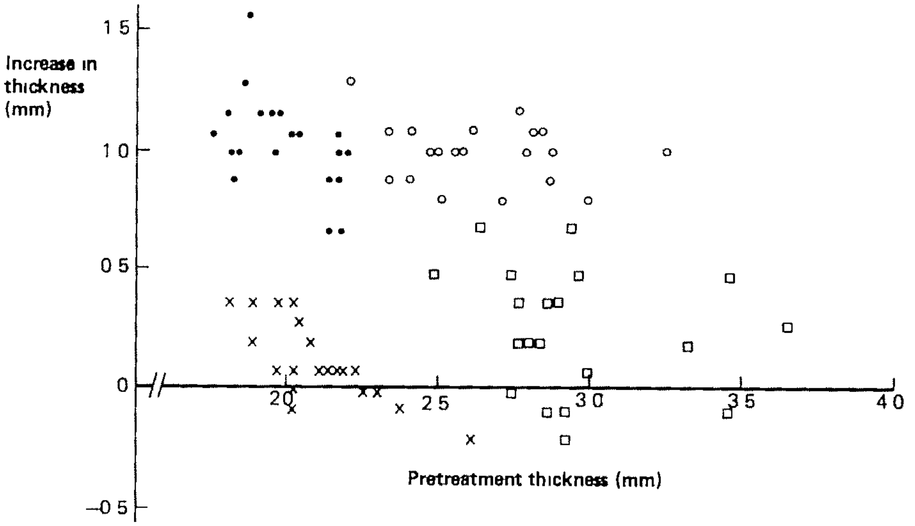

FIGURE 2. Relationship of pre-treatment skin thickness and increase in skin thickness in four different patients. Subjects ● and x showed an approximately linear relationship, subjects ○ and □ did not show a linear relationship.

allowing for the possible linear relationship with pre-treatment thickness. This gave adjusted values for the mean increase in skin thickness, calculated assuming a common pre-treatment thickness of 2·3 mm, the mean value, throughout.

Although this analysis showed that pre-treatment skin thickness had a significant effect on the increase in thickness, the adjusted values still showed a significant variation between sites, and the adjusted mean values for increase in skin thickness differed little from the unadjusted means (Fig. 1c). However, the adjusted values did not show a rise from wrist to elbow although the slightly larger increases in skin thickness on the right arm and on lateral sites near the wrist was found. As with the unadjusted values, the adjusted values for increased skin thickness did not vary significantly with the patients' age, sex or skin type.

## DISCUSSION

We have shown that clinically normal volar forearm skin is thicker distally and laterally than proximally and medially and that the inflammatory increase after anthralin application varies significantly with application site and is greater proximally, laterally, on the right arm and when pre-treatment thickness is low. Although the magnitude of these differences is not great, some are clinically important since they are greater than the 0·1 mm resolution of calipers (Edwards *et al.*, 1955), and greater than 0·2 mm which would be considered significant in a study of anthralin inflammation. Reasons for this variation are not known, but these differences make it impossible to identify a group of sites with a sufficiently uniform response that they can be used interchangeably in an experiment. Since the variation is small and more studies would need to be done to establish correction factors for each site which could be used to standardize experimental measurements, the problem is best avoided by use of symmetrical sites and randomized treatments.

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**Pharmacology and Treatment**

# Short-term use of cyclosporin A in severe psoriasis

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## SUMMARY

The effectiveness of cyclosporin A (CyA) in low dosages (mean 5 mg/kg/day) for short-term treatment of severe psoriasis was studied. Of five patients with severe progressive psoriasis vulgaris (mean PASI score 43·8), an almost complete remission in three patients, and a large reduction in PASI score in the remaining two patients, was obtained within 4 weeks. No important clinical side-effects were found but there was biochemical evidence of slight renal dysfunction in one patient. The mean percentage reduction in the PASI score was 84%. It was concluded that the results reported justify further study of the use of CyA in the treatment of severe psoriasis.

In psoriasis a number of aberrations of the immune system have been reported (Baker *et al.*, 1984). The cellular infiltrate in the clinically active psoriatic lesions may contain 30% activated T cells (Baker *et al.*, 1984) and there is evidence of functional defects in T-lymphocytes (Gladman *et al.*, 1981).

Cyclosporin A (CyA) may interfere with T-cell functions, i.e. by blockade of interleukins, in particular interleukin-2 (Bendtzen, 1984). Apparent responsiveness of psoriatic lesions to CyA has been described recently (Mueller & Herrmann, 1979). In a restricted number of patients with renal transplants and psoriasis, it has been reported that the skin lesions improved significantly during cyclosporin treatment (Harper, Keat & Staughton, 1984; van Hooff, Leunissen, van der Staak, 1985). We present here a pilot study aimed at determining the effectiveness of a short-term 'crisis-intervention' treatment of four weeks with CyA in five patients. In addition, the response to CyA was evaluated during a second period of 4 weeks.

## METHODS

### *Patients*

Five patients (Table 1) were selected on the following criteria: unequivocal clinical and

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TABLE 1. Reductions in PASI* scores in five patients with severe progressive psoriasis vulgaris, treated with cyclosporin A

| Patient no. | Sex | Age (years) | PASI score before treatment | PASI score after treatment for: 1 week | 2 weeks | 3 weeks | 4 weeks | Reduction in PASI score after 4 weeks (%) | PASI-score after treatment for 8 weeks |
|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 51 | 53·2 | 23·5 | 8·4 | 4·7 | 0·9 | 98 | 10·3** |
| 2 | M | 73 | 32·3 | 29·8 | 26·4 | 12·0 | 0·9 | 97 | 1·1 |
| 3 | M | 55 | 58·0 | 43·2 | 36·5 | 13·2 | 10·0 | 83 | 8·0 |
| 4 | F | 48 | 28·2 | 13·8 | 13·5 | 12·6 | 12·3 | 57 | 1·3 |
| 5 | F | 40 | 47·5 | 34·8 | 28·4 | 18·0 | 6·5 | 86 | 2·7 |
| Mean | | 53 | 43·8 | 29·0 | 22·6 | 12·0 | 6·1 | 84 | 4·7 |

* PASI = Psoriasis Area and Severity Index (Fredriksson & Petterson, 1978).

** Cyclosporin not given in weeks 5 and 6.

histopathological diagnosis of chronic plaque form psoriasis, severe disease, long duration of disease (mean 27 years), and resistance to conventional anti-psoriatic therapy (topical therapy including steroids, systemic therapy with PUVA or retinoids, or both). Four patients had been treated previously with methotrexate with variable results.

On entering the study, all the patients exhibited progressive psoriasis with serious physical and social disabilities. Patients were excluded from the study if they were pregnant or suffering from infection, malignancy, impaired renal function (serum creatinine > 110 μmol/l), impaired liver function, uncontrolled hypertension (age 18–50 RR > 150/95, age 50–60 RR > 160/100), epilepsy, malabsorption syndrome, or drug or alcohol abuse. Other criteria for exclusion were: treatment with methotrexate, retinoid or PUVA within one month, or topical anti-psoriatic agents within 2 weeks before the start of the trial.

Clinical evaluation before and during therapy was carried out using the Psoriasis Area and Severity Index (PASI) (Fredriksson & Petterson, 1978). The PASI score increases in units of 0·1 from 0·0: no lesions, to 72·0: complete psoriatic erythroderma of the severest possible degree. The PASI score was recorded before the start of the trial and at weekly intervals during therapy.

On entering the study all five patients had a PASI score $\geqslant 28$ (mean 43·8) (Table 1). Cyclosporin A (CyA) (Sandimmune®, 100 mg/ml) was given orally in two daily doses on the basis of body weight. Full blood count, renal and liver function and blood pressure were measured at weekly intervals. Determinations of trough blood CyA concentrations using a Cyclosporin RIA-Kit, (Sandoz Ltd, Basel, Switzerland) were performed weekly (Table 2).

The initial dose of CyA (4·4–6 mg/kg/day) was given for 4 weeks (Table 2). In the second 4-week period (weeks 5–8) the dose of CyA was gradually adapted for individual patients, in particular in relation to the PASI score (Table 2). No other anti-psoriatic treatment was given.

## RESULTS

The PASI scores in the patients before entering the trial and after 1, 2, 4 and 8 weeks are given in

TABLE 2. Doses of cyclosporin A (mg/kg/day) and trough blood levels (ng/ml) (in parentheses) in five patients with severe progressive psoriasis

| | Weeks 1–4 | | | | Weeks 5–8 | | | |
|---|---|---|---|---|---|---|---|---|
| Patient no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1 | 6 (600) | 6 (720) | 4·7 (770) | 4·8 (890) | ** (120) | ** (60) | 4·7 (530) | 4·7 (590) |
| 2 | 5·6 (700) | 5·6 (670) | 5·6 (650) | 4·4 (610) | 4·4 (610) | 3·9 (—) | 3·6 (—) | 3·6 (—) |
| 3 | 5·7 (—) | 5·7 (390) | 5·7 (430) | 5·0 (450) | 5·0 (480) | 5·0 (520) | 4·3 (250) | 4·3 (160) |
| 4 | 5·5 (530) | 5·5 (460) | 5·5 (490) | 5·5 (430) | 7·0 (410) | 8·1 (370) | 8·1 (750) | 7·4 (980) |
| 5 | 5·0 (210) | 5·0 (210) | 5·0 (250) | 5·0 (250) | 5·0 (240) | 4·4 (—) | 4·4 (170) | 4·4 (70) |
| Mean | 5·6 (510) | 5·6 (530) | 5·3 (518) | 4·9 (526) | 5·4 (372) | 5·4 (317) | 5·0 (400) | 4·9 (450) |

** In Case 1 cyclosporin was stopped during weeks 5 and 6 (*see* text).

— = Not estimated.

Table 1. The dosages and trough blood concentrations of CyA are given in Table 2. Mean PASI scores, trough blood CyA concentrations and CyA dosages over weeks 1–4 are shown in Figure 1.

### *First treatment period (weeks 1–4)*

A low dose of CyA (mean 5 mg/kg/day, Table 2) had a satisfactory effect in all five patients with severe psoriasis. In Patient 1 a substantial fall in the PASI score was found within the first 2 weeks (53·2–8·4). In Patients 4 and 5, within 2 weeks, the PASI scores fell by 53% and 40% respectively. The mean PASI score in the five patients after 2 weeks was 22·6 compared with 43·8 at start of the trial. The mean PASI score fell from 43·8 to 6·1 in the first 4 weeks of the treatment. This represents a mean improvement of 84%. The clinical response by the end of the first 4 weeks was most striking in Patients 1 and 2 (98% and 97% reduction in PASI score respectively, Table 1).

### *Second treatment period (weeks 5–8)*

The decision was made to stop CyA in Patient 1, during weeks 5 and 6, because of the striking remission (PASI score: 0·9). This resulted in an acute relapse within one week. Thereafter, the psoriasis reacted far more slowly to re-institution of CyA at a dose of 4·7 mg/kg/day (trough blood level: 530–590 ng/ml) (PASI score at 8 weeks: 10·3; Table 1). In Patient 4, after increasing the dosage to 7·0 mg/kg/day (Table 2), there was a fall in the PASI score from 12·3 (week 4) to 1·3 (week 8). In the second period of 4 weeks the mean PASI score did not change substantially; falling from 6·1 to 4·7. During this period of the trial a low dose of CyA was found to be effective, giving excellent results in particular in Patients 2 and 4. In Patients 2, 3 and 4 a substantial improvement occurred in psoriatic nail lesions. In Patient 4, with severe psoriatic arthropathy, a striking subjective and objective improvement was noticed ($\geqslant$40%).

Clinical side-effects, including mild reversible hypertension (160/100) (Patients 1 and 4), and mild intermittent diarrhoea (Patient 3), were observed. Clinically important disturbances of renal or liver function or blood count were not seen. Nevertheless, biochemical evidence of mild reversible renal dysfunction was found in one patient (Patient 4).

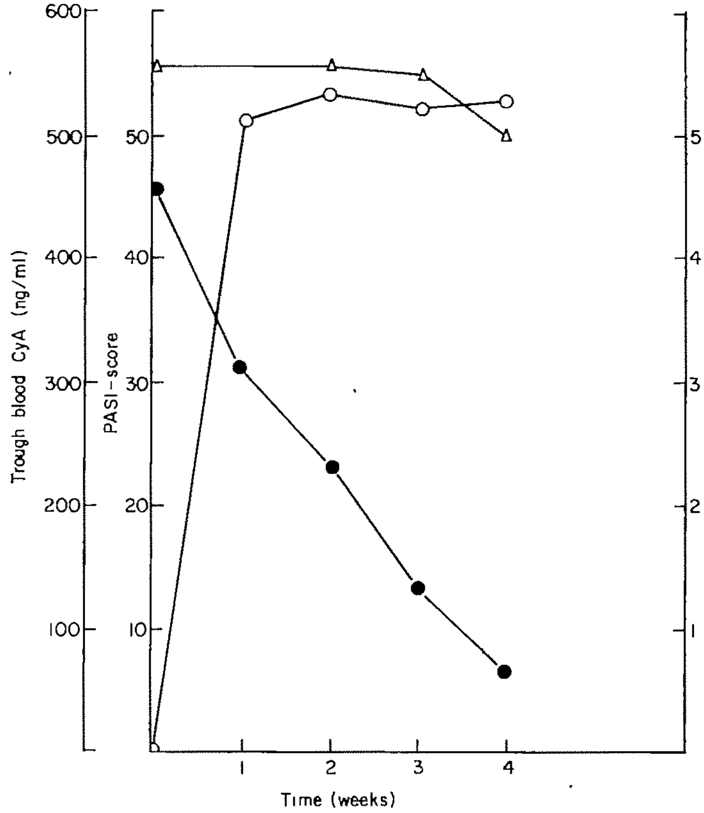

FIGURE 1. Mean CyA dose, trough blood CyA level and PASI score in five patients with severe psoriasis. ● Mean PASI score; ○ mean trough blood level of CyA (ng/ml); △ mean dosage of CyA.

## DISCUSSION

We report here the effectiveness of cyclosporin A (CyA) in crisis intervention therapy in five selected patients with severe, chronic and drug-resistant psoriasis. Mueller & Herrmann (1979) first reported a dramatic response to CyA within one week in four psoriatic patients but the psoriatic lesions gradually returned to their previous severity within 2 weeks after stopping CyA. Müller and Graf (1981) described four patients who received CyA with a striking improvement of severe psoriatic arthritis and of psoriatic skin lesions. These authors observed improvement of the arthritis in three out of the four cases and a striking reduction of the psoriatic skin lesions in all four cases in the first 2–4 weeks of treatment with CyA in high initial doses (about 900 mg/day). Three out of the four cases relapsed after the dosage of CyA was reduced 200–400 mg/day. In these studies no measurements of the clinical improvement in relation to blood concentrations of cyclosporin were performed.

In our patients a tendency to a dose-dependent response to CyA was seen in Patients 1 and 4. In Patient 3 a less striking response was seen (PASI score: 8·0 at week 8) with lower trough blood concentrations of CyA (160–250 ng/ml). In Patient 5, however, despite a very low blood concentration (70 ng/ml at week 8, Table 2), the PASI score remained low (2·7).

The number of patients was too small for adequate experiments to determine optimal dosages. However, the present study shows that at dosages between 4·7 and 8·1 mg/kg/day (trough blood concentrations between 390 and 980 ng/ml), short-term therapy with cyclosporin A is of value as psoriasis 'crisis intervention'.

The pharmacokinetic mechanism in psoriasis is still unknown, but it is tempting to assume that modification of T-cell functions by CyA plays a central role in the response to CyA treatment. However, further studies are required to investigate possible inhibitory effects of CyA on the abnormal proliferation of keratinocytes in psoriasis. Keratinocytes produce a cytokine (epidermal cell derived thymocyte-activating factor) which closely resembles interleukin-1 (Sauder *et al.*, 1982).

CyA acts in the preliminary phase of the immune response and it has been suggested that if long-lived populations of primed T-cells are operative, the beneficial response to the drug may become apparent after several months of treatment (Editorial, 1985). Such a mechanism would explain the incomplete responses in our patients.

CyA can give rise to a variety of side-effects and, for the present, its use—like that of other immunosuppressants—must be contemplated only in severe psoriasis, which is physically or socially extremely disabling. Treatment of psoriasis and psoriatic arthritis with cytostatic drugs as methotrexate can also evoke a beneficial response within a reliable period, but numerous side-effects (in particular of the liver and bone marrow) are described (Oppolzer, Diem and Wolff, 1976). We obtained a mean reduction in the PASI score of 84% after 4 weeks of treatment. Fredriksson and Petterson (1978) reported a reduction of 63% after 4 weeks' treatment with retinoids in a group of 10 patients with severe psoriasis. A few possibilities were considered after the eighth week of treatment: (a) stopping CyA treatment; (b) trying a low maintenance dosage for a longer time and, (c) a low maintenance dosage of CyA in combination with other anti-psoriatic treatment. In our patients we decided after the eighth week to continue a low maintenance dose of CyA with no other treatment and further individual decreases in dosage, but maintenance of blood concentrations of ⩾200 ng/ml. In Patients 2 and 4, therapy with mean dose of 4 mg/kg/day was continued for a further 5 and 12 weeks respectively and was then stopped (PASI score in both cases was 0 at this point). In Patient 2 the PASI score remained 0 two months after stopping CyA, without any other therapy. Patients 1 and 5 are still under study. In Patient 3 (PASI score of 8 at week 8) the PASI score was reduced nearly to 0 after 10 further weeks on CyA at a dosage of 4·5–5 mg/kg/day (trough blood concentration ⩾200 ng/ml). In this case, however, a local relapse of the skin lesions on the hands occurred when the dose of CyA was reduced below 4 mg/kg/day.

The results of the present study would appear to justify further controlled studies on the use of CyA in severe psoriasis.

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# Iron removal therapy in porphyria cutanea tarda: phlebotomy versus slow subcutaneous desferrioxamine infusion

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## SUMMARY

Twenty-five patients with overt clinical and biochemical findings of porphyria cutanea tarda took part in a study comparing intensive phlebotomy with slow subcutaneous desferrioxamine treatment. Fifteen male patients (Group A) had intensive venesection therapy. Ten patients (Group B) with associated diseases (minor thalassemia, cardiovascular impairment, pulmonary tuberculosis or severe liver cirrhosis) received 1·5 g of desferrioxamine by slow subcutaneous infusion using an automatic syringe pump 5 days a week. No patient complained of appreciable side effects. Serum iron, ferritin and uroporphyrins were normalized in all subjects by the end of treatment. The mean time necessary for complete recovery was 13·8 months (range 9–19) and 11·2 months (range 6–14) in Groups A and B, respectively. Liver function significantly improved during and after the treatments in both groups. We conclude that recovery from porphyria cutanea tarda can be achieved equally well using phlebotomy or desferrioxamine subcutaneous infusion. Phlebotomy is easily performed and remains the treatment of choice; slow subcutaneous desferrioxamine treatment, although expensive, is recommended when severe associated diseases contra-indicate venesection.

Porphyria cutanea tarda (PCT), the most common of the hepatic porphyrias, results from a genetic disorder of uroporphyrinogen-decarboxylase (UD), the enzyme responsible for the conversion of uroporphyrinogen into coproporphyrinogen (Kushner, Barbuto & Lee, 1976; Kappas, Sassa & Anderson, 1983). The liver is the main site of uroporphyrinogen production (Elder, Lee & Tovey, 1978; Felsher *et al.*, 1982), although the same enzyme defect has been reported also in red blood cells and in other tissues in the genetically determined form of the disease (de Verneuil, Aitken & Nordmann, 1978). Abnormal iron metabolism, resulting in

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hepatic siderosis, is often associated with liver disease (Cortes *et al.*, 1980; Lefkowitch & Grossman, 1983) and is thought to contribute to the pathogenesis of PCT, although the pathway is not yet fully understood (Baravalle & Prieto, 1983). Other factors can be involved also in the pathogenesis of PCT such as hydrochlorinated hydrocarbons (Cripps *et al.*, 1984) and dioxin (Doss *et al.*, 1984); in recent years the disease has become more frequent in women receiving oestrogen therapy (Taylor & Roenigk, 1976) and in patients undergoing long-term dialysis (Poh-Fitzpatrick, Masullo & Grossmann, 1980).

However, iron overload, sometimes related to alcohol excess, clearly plays an important role in inhibiting UD activity (Kushner, Steinmuller & Lee, 1979; Mukerji, Pimstone & Burns, 1984), and iron removal procedures generally prove effective in the treatment of PCT (Ippen, 1977). Support for the hypothesis that iron plays a key role in PCT comes from the finding that the recovery from the cutaneous and biochemical symptoms of PCT normally obtained with repeated phlebotomies is prevented by iron supplementation (Lundvall, 1971), while the addition of chelating agents to cell cultures prevents the inhibition by iron of UD activity (Kushner *et al.*, 1979). Chloroquine cannot be considered an alternative choice to iron removal in patients with liver damage (Cainelli *et al.*, 1983). Furthermore, it has been suggested recently that one or more unidentified substances in the bloodstream (removable by plasma exchange) may stimulate uroporphyrin synthesis (Disler *et al.*, 1982). However, the treatment of choice for PCT is still normally based on removal of iron, and the usefulness of repetitive venesections is well documented (Ippen, 1977).

Severe liver damage, haemolytic or hyporegenerative anaemias, cardiovascular impairment and other associated diseases may contra-indicate frequent blood loss (Gibertini *et al.*, 1984a). We tested, therefore, the efficacy of long-term subcutaneous infusion of desferrioxamine (DFX) as a possible treatment of PCT (Gibertini *et al.*, 1984b).

In the present study the different therapeutic regimens (venesection versus DFX infusion) were compared in two groups of PCT patients.

## METHODS

Twenty-five patients (23 male, 2 female) aged 40–63 years (mean 52 years) took part in the study. All subjects presented with the typical skin lesions resulting from PCT (skin fragility, bullae and crusting on sun-exposed areas). Biochemical diagnosis was based on urinary levels of porphyrins, detected as free acids by high-performance liquid chromatography, which allows a clear separation of the various carboxylic compounds (Gaetani *et al.*, 1982). Urine concentrations of delta-aminolaevulinic acid and porphobilinogen were also measured (Davis & Andelman, 1967). Liver pathology was defined on the basis of routine biochemistry according to standard methods (aminotransferases, serum albumin and globulins, prothrombin time, alkaline phosphatase, $\gamma$-glutamyl-transferase, bromosulphthalein retention and bilirubin), as well as liver radioisotopic and echographic scans and needle liver biopsy. All patients were tested for hepatitis B virus markers and alpha-feto protein levels (RIA). Serum iron, transferrin saturation and serum ferritin (RIA) were taken into account in detecting iron overload, together with Perls' stain of the liver biopsy (Scheuer, Williams & Muir, 1962).

Patients were divided into two groups:

*Group A.* Fifteen PCT patients with only minor liver damage and without impairment of cardiovascular, renal or haemopoietic function underwent repeated venesections. A 300 ml phlebotomy was performed weekly or twice weekly until serum iron fell below 10·7 $\mu$mol/l, haemoglobin below 11 g/dl and serum ferritin and urinary excretion of porphyrins were almost halved. Thereafter, blood was let monthly or bimonthly until complete recovery.

*Group B.* Ten PCT patients with associated severe liver disease or cardiovascular impairment, haemolytic or hyporegenerative anaemia, or with a recent history of tuberculosis, were treated with an iron chelating agent, desferrioxamine (DFX) (Desferal®, Ciba-Geigy, Basel, Switzerland). The drug (1·5 g dissolved in 8–10 ml of sterile saline) was infused subcutaneously using a small portable, battery powered syringe pump (Cx type, Carandina Olivia Lab, Bondeno, Italy) programmed for 8–10 h. The infusion was given 5 nights a week. Urinalysis, serum electrolytes (Na, K, Ca, P, Cu), renal function, liver function, serum iron, serum ferritin and urinary porphyrins were checked monthly after DFX treatment was started. When urinary excretion of porphyrins reached one-third of the initial value (or was considerably decreased) the schedule of DFX infusions was reduced to 5–10 days every month or every other month. Urinary excretion of iron was also determined in each patient.

### *Statistical analysis*

Results in the two groups were compared using an analysis of variance. Measurements within each group before and after treatment were analysed using a paired *t*-test.

## RESULTS

The two groups differed in the severity of liver damage and in other pathological features, but they were comparable with respect to age (mean ± SD, 51 ± 6 and 52 ± 6 years, respectively), alcohol consumption and porphyrin excretion. Their biochemical findings are listed in Table 1.

Liver histology, in particular with respect to evidence of iron overload, is listed in Table 2. Most patients had hepatic siderosis graded histologically from mild to severe (Scheuer *et al.*,

TABLE 1. Laboratory findings in PCT patients

| | Group | |
|---|---|---|
| | A | B |
| Bilirubin (μmol/l) | 15·3 ± 5·1 | 15·4 ± 5·3 |
| AST (IU/l) | 41 ± 18 | 63 ± 31 |
| ALT (IU/l) | 66 ± 32 | 103 ± 41 |
| γ-glutamyl-transferase (IU/l) | 65 ± 44 | 114 ± 111 |
| Bromosulphthalein retention 45′ (%) | 10·8 ± 7·6 | 14·6 ± 7·5 |
| Prothrombin time (%) | 89 ± 7 | 88 ± 8 |
| Serum albumin (g/l) | 36 ± 3 | 38 ± 3 |
| Serum γ-globulin (g/l) | 13 ± 4 | 14 ± 4 |
| Serum iron (μmol/l) | 31·3 ± 5·7 | 30·8 ± 9·8 |
| Transferrin saturation (%) | 51·8 ± 15·3 | 48·5 ± 13·2 |
| Serum ferritin (μg/l) | 631 ± 407 | 539 ± 238 |

Figures are means ± SD for 15 patients in Group A and 10 patients in Group B.

1962), and iron deposition was mainly in hepatocytes rather than in Kuppfer cells. Hepatitis B virus surface antigen was detectable in two patients in Group A; antibodies against surface and core antigens were present in seven patients in Group A and in four in Group B.

### *Group A*

The number of venesections required for complete recovery from cutaneous and biochemical symptoms of PCT ranged from 15 to 32 (mean ± SD, 21 ± 4) over a period of 9–19 months (mean ± SD, 13·8 ± 3·3). No patient experienced severe side-effects. Mean uroporphyrin levels fell from 1990 ± 651 μg/g urinary creatinine to 48 ± 26 μg/g. Serum iron and ferritin reached normal levels, decreasing from a mean of 31·3 ± 5·7 to 13·0 ± 3·9 μmol/l and from 631 ± 407 to 53 ± 49 μg/l, respectively. At the end of the therapy serum albumin levels were found to be significantly improved, and aminotransferase levels reduced. Changes in albumin, aminotransferase, serum iron, ferritin and uroporphyrin in the individual patients are shown in Fig. 1. Levels of all these compounds had changed significantly at the end of the treatment.

### *Group B*

The patients' compliance with DFX treatment was good and the infusions were locally well tolerated. Periodic ophthalmic examination excluded cataracts and retinal degeneration (Davies *et al.*, 1983); no clinical signs of oligo-element depletion were observed.

The period required to achieve normalization of biochemical and cutaneous signs of PCT ranged from 6 to 14 months (mean ± SD, 11 ± 2). Mean uroporphyrin levels fell from 1917 ± 684 to 29 ± 13 μg/g urinary creatinine, serum iron fell from 30·8 ± 9·8 to 17·5 ± 4·6 μmol/l and serum ferritin fell from 539 ± 238 to 88 ± 79 μg/l. Liver function also improved in these patients and a reduction in serum aminotransferase and an improvement in serum albumin levels were also observed (Fig. 1). Comparison of the levels of these compounds before and after treatment showed that all had changed significantly.

Statistical comparison between the two groups (using ANOVA) showed a significant difference only in the case of initial aminotransferase levels, which were higher in Group B ($P < 0{\cdot}05$). Moreover, the mean length of the two treatments did not differ significantly, although that for Group B was slightly shorter.

TABLE 2. Features of hepatic histology in PCT patients

| | No. of patients | |
|---|---|---|
| | Group A | Group B |
| Total patients | 15 | 10 |
| Infiltration | 13 | 9 |
| Necrosis | 9 | 8 |
| Fatty change | 8 | 6 |
| Siderosis (+ +, + + +) | 10 | 7 |
| Architecture: | | |
| distorted | 5 | 4 |
| cirrhotic | 3 | 4 |

## DISCUSSION

All treatments of PCT begin with abstinence from alcohol and withdrawal of oestrogens; both hepato-toxins which are considered important factors in triggering the clinical disease (Topi, Amantea & Griso, 1984). However, a full remission cannot usually be obtained simply through the avoidance of toxic factors once the overt clinical disease is present. Iron removal therapy is the therapy of choice and there have been several biochemical demonstrations of the pathogenetic role of iron. Since Ippen (1977) first demonstrated the efficacy of phlebotomy, its safety and results have been widely confirmed (Ramsay *et al.*, 1974; Kappas *et al.*, 1983).

As venesection may be contra-indicated in some patients (Gibertini *et al.*, 1984a), we tested the efficacy of DFX treatment, given by means of subcutaneous infusion to overcome the short half-life of the drug (Summers *et al.*, 1979). Since therapy with DFX is rather expensive, it cannot be advocated for patients able to tolerate venesections, at least until its effectiveness has been verified.

We consider the proposed alternative, chloroquine (Ashton, Hawk & Magnus, 1984), a hazardous choice on account of its toxicity for liver mitochondria and the fact that centrolobular hepatocyte necrosis has been demonstrated histologically (Graifemberghi *et al.*, 1982; Cainelli *et al.*, 1983), especially because all our PCT patients showed histological signs of liver damage.

Cutaneous and biochemical symptoms of PCT resolved in a comparable period of time in each group. However, in Group B patients, melanodermia, bullae and fragility of the skin improved within 2–3 months of treatment, even before uroporphyrin levels had consistently fallen, while in the phlebotomized patients (Group A) mucosae and skin became paler as a result of transient anaemia, while photosensitive cutaneous lesions were evident for a longer period. By chelating the iron from the intracellular pool, which is directly linked to the biologically active pool (free toxic iron), DFX immediately reduces the amount of intracellular free radicals, which are responsible for stimulating melanin synthesis (Graziano, 1976).

Subcutaneous infusion of DFX was found to be as effective as repeated phlebotomies in normalizing porphyrin excretion and iron storage. In fact, ferritin, the most important laboratory index of liver iron stores (Rocchi *et al.*, 1986), normalized equally well with both treatments, although serum iron levels remained slightly higher (but within the normal range) in Group B. Liver function, too, was improved by removal of iron in all patients, a reduction in aminotransferases being more evident in Group B patients, who also had more severe liver disease.

Compliance with the two therapies was good. No patients stopped the treatment, even though several Group A patients complained of feeling mildly debilitated, mostly during the initial stages of therapy when venesections were performed weekly, so that they were forced to restrict their work. Group B patients, on the other hand, had only transient erythema and minimal pain at the site of injection, once trained in the correct use of the pump; all patients in this group were able to continue normal work, since infusions were carried out at night.

There was no apparent difference between the familial and sporadic form of the disease with regard to the response to either of the treatments. Hepatic siderosis is found in nearly all patients and iron overload is involved in the pathogenesis of the disease irrespective of the form (Kushner *et al.*, 1985). However, UD activity in red blood cells was not measured in all cases, and so no definite conclusion can be drawn.

The results of DFX treatment are not consistent with the presence of a circulating factor stimulating porphyrin synthesis, the removal of which may be obtained by plasmapheresis (Disler *et al.*, 1982; Miyauchi, Shiraishi & Miki, 1983). DFX removes mainly iron, the

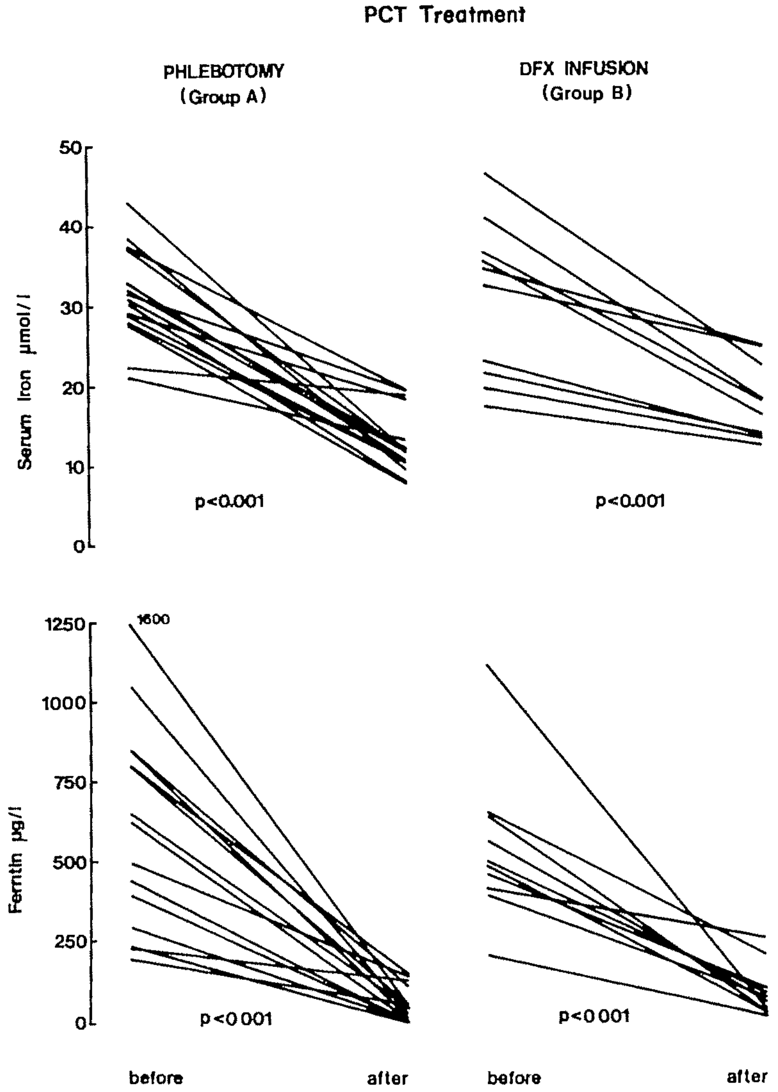

FIGURE 1. Serum iron, ferritin, urinary uroporphyrin, serum alanine aminotransferase and albumin in each patient before and after treatment. (Continued on facing page).

pathogenetic role of which is well documented (Mukerji *et al.*, 1984), and also minor quantities of other low mol. wt. compounds (Zaino, 1977), but does not remove any non-dialysable factors. We believe that further studies are needed to verify whether a low mol. wt. compound, chelated by DFX, is involved in stimulating porphyrin synthesis, either directly or through the supposed 'stimulating factor'.

We would confirm that iron removal should be therapy of choice in PCT and conclude that phlebotomy and subcutaneous infusion of DFX achieve similar results, while also improving liver function. Phlebotomy is less expensive and easier to perform and is thus the mainstay in

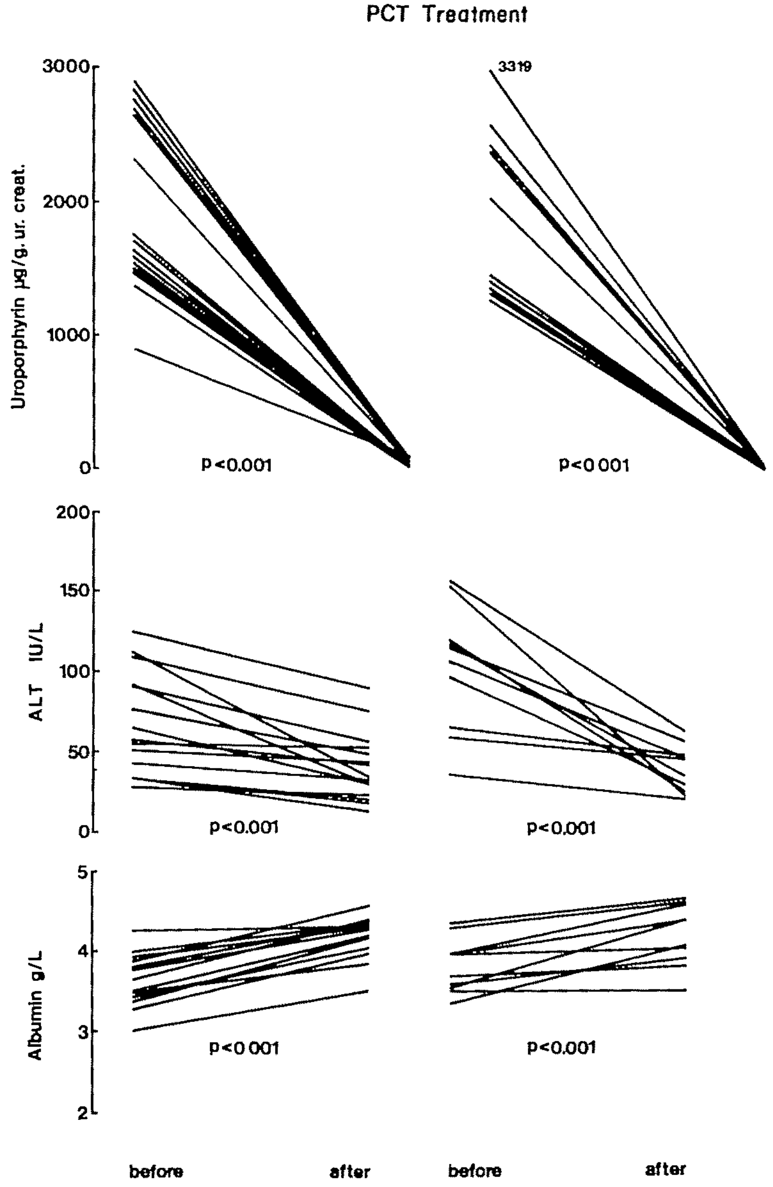

FIGURE 1. (Continued).

iron overloaded patients. However, anaemia or other major clinical problems may prevent general use. Since DFX treatment is very expensive, the decision on whether or not to start chelation must be based on the degree of iron overload and the risk of undergoing repeated blood losses.

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***Case Report***

# Giant orf in a patient with chronic lymphocytic leukaemia

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## SUMMARY

A case of orf is presented in a patient with chronic lymphocytic leukaemia treated with chlorambucil and prednisone. Diagnosis was confirmed by electron microscopy and histopathology. Human orf is normally a self-limiting and benign condition but in this case the lesion grew to a size of 60 × 60 × 28 mm. When it was excised, local recurrence was treated with topical idoxuridine.

Chronic lymphocytic leukaemia (CLL) may show features of both humoral and cell-mediated immune deficiency. The patients are prone to viral infections, especially with varicella zoster, herpes simplex, cytomegalovirus and adenoviruses (Bodey, Bolivar & Fainstein, 1982). They appear to be prone to warts (Morison, 1975; Blamey, 1968), to widespread molluscum contagiosum (Rimbaud *et al.*, 1969; Ganpule & Garretts, 1971; Rosenberg & Yusk, 1970), and to genital warts (Oriel, 1977). Atypical and severe forms of viral infection are also seen.

In human orf infection, spontaneous regression and healing is usually seen within 7–10 weeks (Hunskaar & Dahle, 1983; Johannesen *et al.*, 1975). This case report presents an opportunistic viral infection due to the ecthyma contagiosum (orf) virus occurring in a patient with chronic lymphocytic leukemia (CLL) which had been in remission but relapsed at the time of orf infection, the first clinical sign of impaired immunological status.

## CASE REPORT

A 55-year-old farmer was referred in October 1981 after he had noticed a lump on the right side of his neck. Two weeks previously the patient had had pneumonia, general lymphadenopathy and hepatosplenomegaly. Past history was gastrectomy in 1956 and surgery for a herniated disc in 1976. The patient's father had died of lymphoma. The diagnosis of chronic lymphocytic leukaemia (CLL) in stage 4 was established in our case. Relevant investigations and results are

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shown in Table I. The patient was treated with prednisone 10 mg daily and chlorambucil 10 mg daily. The patient went into remission and the treatment was discontinued after 6 months.

In October 1982 the patient sheared his sheep and a week later an ulcer appeared in the palm of his right hand, followed by a nodule. The lesion was incised but no pus was found. On examination the tumour was found to measure 20 × 15 × 15 mm. Clinical diagnosis was ecthyma contagiosum, and electron microscopy showed abundant orf viruses (Fig. 1), but he had no evidence of active CLL. The tumour did not undergo remission and remained stable for 6–8 weeks, when it grew rapidly over two weeks to measure 60 × 60 × 28 mm (Fig. 2). The tumour was excised by carefully dissecting epidermis from dermis. It weighed 42 g. Histopathology showed an acanthotic hyperkeratotic epidermis and areas with intracellular oedema and ballooning. The dermis showed areas with oedematous granulation tissue with abundant thin-walled blood vessels. Lymphocytes, plasma cells and macrophages were seen but no viral inclusion bodies or malignant cellular transformation. Three small areas of local recurrence, which appeared as red-blue centres surrounded by a small white ring were treated with topical idoxuridine 40% in dimethylsulphoxide tid for 6 days (Hunskaar, 1984), and disappeared after

TABLE I. Laboratory findings in the patient with orf virus infection and chronic lymphocytic leukaemia. Diagnosis of orf was established in October 1982; the lesion was excised in January 1983

| | Hb g/100 ml | ESR mm/h | WBC × $10^9$/l | Platelets × $10^9$/l | Gel electrophoresis | IgM g/l | Differential count |
|---|---|---|---|---|---|---|---|
| Normal value | 13·5–16·0 | 0–20 | 4–11 | 150–400 | | 0·3–2·5 | Neutrophils 56%<br>Monocytes 4%<br>Lymphocytes 34% |
| *Patient's result* | | | | | | | |
| November 1981 | 9·2 | 30 | 110 | 75 | N | 0·12 | Chronic lymphocytic leukaemia, little differentiation |
| June 1982 | N | N | N | — | N | 0·03 | N |
| December 1982 | 15·6 | N | N | — | N | 0·02 | Neutrophils 36%<br>Monocytes 7%<br>Lymphocytes 57% |
| January 1983 | 13·4 | N | 13·4 | 216 | N | — | Neutrophils 31%<br>Lymphocytes 67%<br>Many undifferentiated |
| February 1983 | 13·9 | N | 28·7 | 162 | N | — | Lymphocytes 70%<br>In marrow film:<br>72% lymphocytes with an immature appearance |
| April 1983 | 13·0 | 5 | 31 | 120 | N | — | Lymphocytes 98%<br>⅓ nucleoli |
| July 1983 | 9·1 | 12 | 60 | 51 | N | — | Lymphocytes 80% |

N = normal.

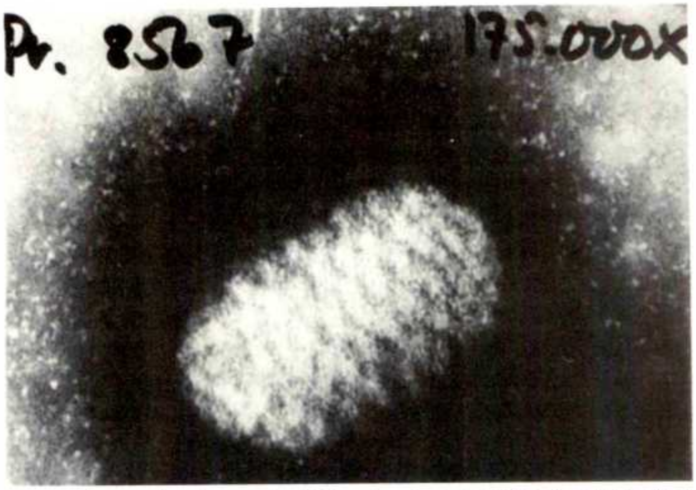

FIGURE 1. Electron-microscopic picture of orf virus in a tissue sample taken from the lesion. (Original × 175 000).

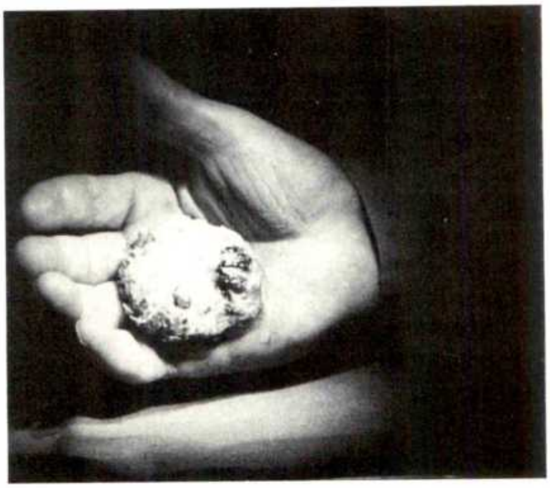

FIGURE 2. Giant orf virus lesion in the patient with chronic lymphocytic leukaemia. The lesion measured 60 × 60 × 28 mm.

14 days. The CLL relapsed in the following months and in July 1983 treatment was started with prednisone and melphalan.

## DISCUSSION

This giant orf lesion appears to be the largest recorded. Diagnosis was made clinically and confirmed by electron microscopy (Fig. 1) and histopathology. An inappropriate clinical diagnosis of a malignancy could lead to the amputation of a finger or hand (Johannesen *et al.*, 1975). Even when the diagnosis is established, amputation has been performed (Savage & Black, 1972). While dissecting the tumour free from the dermis it was not difficult to find the border between the diseased and healthy tissue.

Local recurrences responded to topical idoxuridine 40% in dimethylsulphoxide. The treatment of human orf with idoxuridine has previously been successful (Hunskaar, 1984) and is of value in therapy of human orf infection when the lesions are not too big.

Although total immunoglobulin levels were normal, levels of IgM were reduced consistently after diagnosis of CLL, which might partly explain the increased susceptibility to infection by facultative intracellular pathogens. A case of 'giant' orf of finger in a patient with lymphoma has been reported (Savage & Black, 1972). Appearance of the orf lesion in our patient preceded relapse of his CLL, and immunosuppressive drugs had been discontinued for at least 6 months when the lesion developed. It is therefore unlikely that chemotherapy could have had any significant influence, although it may affect the immune response.

## ACKNOWLEDGMENTS

The author wishes to thank Professor O.D.Lærum for interpretation of the biopsy, and the Department of Microbiology, Haukeland University Hospital for electron microscopy.

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## Ritodrine-responsive bullous pemphigoid in a patient with AIDS-related complex

MADAM, Individuals afflicted with either the acquired immune deficiency syndrome (AIDS) or the AIDS-related complex (ARC) suffer from profound underlying immunological abnormalities including depressed cell-mediated immunity and polyclonal B-cell stimulation. In addition to Kaposi's sarcoma, seborrhoeic dermatitis, extensive folliculitis, oral candidiasis, fungal infection and herpes simplex and zoster infections are all dermatological conditions often present in patients with either AIDS or ARC. To our knowledge, however, only rarely has an autoimmune disease been reported to be associated with either AIDS or ARC (Paciucci & Holland, 1984; Zolla-Panzner, 1984) although autoantibodies such as rheumatoid factor and anti-nuclear antibodies have been detected in the sera of up to 36% of AIDS patients (Zolla-Pazner, 1984).

We wish to report the association of bullous pemphigoid and ARC in a 58-year-old bisexual white male. This patient has been followed since 1983 for chronic generalized lymphadenopathy, severe asthenia, weight loss, antibodies to HTLV-III (kindly tested for by Dr Gallo, NIH, Bethesda MD, U.S.A.), inversion of the OKT4/OKT8 (helper/suppressor) ratio to 0·83, lymphopenia with a selective defect in the OKT4 subset, and polyclonal B-cell activation. In November 1984 dermatological consultation was sought for severe, generalized, anti-histamine resistant pruritus which disturbed the patient's daily activities and his sleep. Clinical examination revealed widespread excoriated erythematous papules measuring 3–10 mm in diameter, as well as a great many scars of similar size and distribution, evidence of prolonged intense itching. In addition, the patient had eosinophilia (16% of 4700 white blood cells); no parasitic or allergic cause was found and several biopsies of lesional and non-lesional skin were therefore performed. Direct immunofluorescence revealed linear deposits of IgG at the dermo-epidermal junction and immunoperoxidase electron microscopy showed specifically that the antibody-antigen complex was located exclusively in the lamina lucida. No circulating anti-dermo-epidermal junction antibodies were detected.

Our patient with ARC was therefore diagnosed as having bullous pemphigoid in its early, papular pre-bullous form, a well-documented entity (Bingham, Burrows & Sandford, 1984; Barker, 1983; Saurat *et al.*, 1978) and this presented us with a serious treatment dilemma. High dose corticosteroids with or without immunosuppressive agents is the treatment of choice (Lever, 1979) but obviously an unacceptable risk in our patient (Shafer *et al*, 1985). Corticosteroids depress cell-mediated immunity and have already been reported to have hastened the development of fatal opportunistic infections in three patients with ARC (Shafer *et al*, 1985). We therefore opted for a trial of ritodrine, a beta-agonist recently reported to be effective in a case of herpes gestationis (MacDonald & Raffle, 1984)) and which presented no threat to the immune competence of our patient. After 4 weeks of treatment at an average daily dosage of 40 mg, the pruritus completely resolved and the patient discontinued the medication only to have the severe itching return. He was again started on ritodrine with the same excellent results to date with a follow up of 10 months. Our observations suggest that bullous pemphigoid, an autoimmune cutaneous disease, can co-exist with ARC and might possibly be secondary to the profound immunological dysfunction of ARC; also that the beta-agonist ritodrine or a similar compound merits consideration as alternative therapy in patients with bullous pemphigoid and contra-indications to corticosteroids.

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## Reply

MADAM, The therapeutic effect of the beta-mimetic drug ritodrine in our patient with pemphigoid gestationis (herpes gestationis) (*British Journal of Dermatology*, 1984, **111**, 630) was striking, although its *modus operandi* was unclear. We were interested to know whether others had observed a similar effect in herpes gestationis but had not considered a therapeutic trial of this drug in other bullous diseases. The report by Levy *et al.*. of its value in suppressing bullous pemphigoid in a patient with AIDS-related complex is extremely interesting and it is, in fact, the only feed-back we have had on this topic. We agree that further evaluation of the clinical and pharmacological effect of ritodrine in pemphigoid and other bullous diseases is called for.

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## The human skin blanching assay—use and abuse

MADAM, The standard method employed by pharmacologists to study relative potency is to compare equipotent concentrations. This is normally done by preparing dose-response curves and comparing either the maximal response or the concentrations required to produce a half maximimal effect. The assay of corticosteroid potency using the blanching test advocated by Haigh *et al.* (*British Journal of Dermatology*, 1985, **113**, 502) is qualitative and may only be used as a rank order test. There is no *a priori* evidence that the use of a non-parametric 'area under the curve' assay is more reliable than measurement at a single specified time.

Using a simple all or none end-point, McKenzie (1962) was able to use the blanching test to study the dose-response relationship in a manner that eliminated observer bias as far as possible. The dose-response relationship has also been measured objectively using colchicine-arrested mitotic rate measurements (Marks, Pongsehirun & Saylan, 1973) and suppression of irritant-induced epidermal thickening (Barnes *et al.*, 1975). Other methods that may provide quantitative information on corticosteroid-induced effects are measurements of dermal thickness using ultrasound and of skin blood flow.

The blanching test is difficult to interpret since the degree of pallor depends on factors such as ambient temperature and may be obscured by changes in skin colour induced by ointment bases (Burdick, Poulsen & Place, 1970; Coldman & Lockerbie, 1971; Woodford & Barry, 1973). Furthermore, the method is subjective and can only, at best, be used to establish a rank order of potency. Since the blanching test was first introduced, standards of drug assay have improved and the blanching test should no longer be accepted as adequate except as a preliminary crude assay.

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## Reply

MADAM, Whilst we are in agreement with Dr Gaylarde's comment that there is a base induced blanching response in human skin, this normally occurs for only 1–2 h after the removal of the corticosteroid formulation. As we pointed out in our original letter, this is one of our objections to conclusions drawn from single readings of blanching made 1 h after removal of the residual topical formulation.

It is a well established fact that a response-time profile, from which the area under the curve value is determined, provides complete characterization of the observed phenomenon. Considering that the profiles of two topical corticosteroid formulations may be coincident at certain time intervals while deviating substantially at others, the use of a single point assessment may result in an invalid conclusion.

We accept that the human skin blanching assay is subjective in that a visual determination of blanching is made. It should be noted, however, that workers in Germany (Altmeyer & Krumney, 1978) and England (Feather *et al.*, 1982), who have developed quantitative instrumental reflectance methods for measuring skin colour at the site of blanching, have shown a very close correlation between visual and instrumental determinations of the intensity of skin pallor.

We disgree strongly with Dr Gaylarde's statement that the blanching assay should no longer be accepted as adequate. While the simple all or none end point used by McKenzie (1962) may well be considered a preliminary crude assay, considerable research has been performed since that time utilizing and refining this assay in keeping with the general trends of improvement in drug analysis methods. Comprehensive studies by Barry and Woodford (1978) have shown that, provided the protocol of the method is strictly adhered to by experienced workers, the blanching assay is sensitive, accurate and reproducible.

Finally, we would like to assure Dr Gaylarde that we do not advocate the use of the human skin blanching assay. There are several other *in vivo* methods for determining corticosteroid activity which will provide equally meaningful results. What we *are* advocating is that if the human skin blanching assay is going to be used, then it should be used properly.

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## Relative potencies of topical steroids

MADAM, We read with great interest the letter by Haigh *et al.* (*British Journal of Dermatology*, 1985, **113**, 502) concerning the prediction of differences in potency of topical corticosteroid formulations utilising a single reading of the blanching produced during vasoconstrictor assay studies.

We agree that a multiple reading technique during the test procedure is the most finely tuned method of predicting differences between these formulations (Gibson *et al.*, 1984) and clearly it is the only way to produce a blanching response-time profile. However, we do not agree that the obvious superiority of the above method forces the conclusion that the single reading method is invalid. As in any scientific exercise, the methods used and results obtained have to be related to the questions one is trying to answer and the degree of precision required in order to do so. With regard to the vasoconstrictor assay, the key question is whether a single reading method can reproducibly supply information of relevance to clinical dermatological practice. The answer is that it can, provided that the limitations of the method are understood and that data so obtained are interpreted carefully, bearing in mind the possibility of false positive or negative results with any bioassay. In evaluating the relevance of a particular method to dermatological practice it is clearly necessary to validate pioneering work in this field by performing carefully controlled clinical studies. Cornell and Stoughton (1985) have shown recently that there is an excellent degree of correlation between a single reading technique of the vasoconstrictor assay and results obtained from carefully performed double-blind clinical studies, although it has been suggested that in the few cases where correlation did not occur, the more finely tuned multiple reading technique may have helped to clarify the picture (Gibson, 1985). With regard to our own work, one of the key messages which we expressed in our early publications (Gibson *et al.*, 1982; Gibson *et al.*, 1982; Kirsch *et al.*, 1983) and which we still feel to be not only clinically relevant, but supported by subsequent data (Gibson *et al.*, 1984) is that medical practitioners who utilise either extemporaneous or proprietary dilutions of a standard preparation should be aware that their potency may be considerably greater than they anticipate. It is true that in our programme of work the multiple reading technique subsequently yielded more precise information, but results from the single reading technique were accurate enough to justify the publication of the broadly stated cautionary messages. With regard to serial dilutions of corticosteroid ointments in their recommended diluents, we have found that the general profile and shape of the blanching response-time curve remains remarkably consistent and it is therefore quite possible to draw meaningful conclusions from single readings of the vasoconstrictor assay in the case of such dilutions. Conversely, there is no doubt that in order to make correct deductions from comparisons between different corticosteroid formulations, it is important to be aware of the general shape of the curve obtained from multiple readings with each preparation in question. As we have previously pointed out, this requirement is highlighted by such examples as clobetasone butyrate 0·05% ointment (Eumovate) with which relatively high early reading values are followed by rapid weakening of the blanching response (Gibson *et al.*, 1984).

Finally, with regard to the specific objections raised by Haigh *et al.*, we remain unconvinced that the weak blanching produced by various 'inactive' preparations significantly interferes with the sensible interpretation of data obtained during vasoconstrictor assay studies, although there is no doubt that minor degrees of blanching do occur with such preparations. In any event, in the multiple reading technique of the vasoconstrictor assay, if Haigh *et al.* are correct, a similar degree of error would occur in the early readings and this weakens the argument used to justify their statements. Clearly, the total time between application of materials and taking the reading is a highly relevant factor, as corticosteroids take several hours to produce a blanching response. This total consists of the period during which the test materials are in place on the skin and the interval of time after their removal until the first reading has been made. In the single reading technique which we used, assessments were made 7 h are application of test materials (including 1 h after their removal) and in a large series of vasoconstrictor assays we have not encountered any particular difficulty in performing the readings. Furthermore, extremely good correlation was found between results obtained by the three independent scorers who performed their assessments under double-blind conditions. The excellent correlation between results produced by experienced readers of the vasoconstrictor assay, and the reproducibility of the technique following visual assessment, have convinced us that more sophisticated methods of assessment are unlikely to be superior to the relatively simple but reproducible technique which we have utilized.

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## Inflammation of actinic keratoses induced by cytotoxic drugs

Madam, A 63-year-old Caucasian man was undergoing chemotherapy for acute myeloblastic leukaemia. He received four courses of cytotoxics, each course comprising cytosine arabinoside, 180 mg intravenously twice daily on Days 1–5, 6-thioguanine, 160 mg orally twice daily on Days 1–5, and doxorubicin, 90 mg as an intravenous bolus on Day 1.

Following the second, third and fourth courses, on the eleventh, tenth and eighth days respectively, he developed an erythematous maculo-papular eruption with a slight scale. The eruption was distributed over the face, trunk and limbs, sparing only the bathing-shorts area, axillae, palms and soles. The appearance of the lesions was identical to that found in patients with actinic keratoses receiving treatment with topical 5-fluorouracil. The affected areas showed chronic sun damage. The patient was a keen sunbather, but had not exposed the affected areas to the sun since admission. Dilute fluocinolone acetonide ointment provided symptomatic relief, and the lesions faded over the following 2 weeks.

Although the patient had recieved many other drugs during this period, only the cytotoxics could be linked temporally with the separate attacks of inflammation.

Histologically, one of the erythematous lesions showed an atrophic actinic keratosis with a mild superficial and deep lymphohistiocytic perivascular infiltrate. In one area there was a suprabasal cleft associated with acantholytic and dyskeratotic cells in the lower levels of the epidermis.

This reaction has been described previously only in association with the systemic administration of 5-fluorouracil (Falkson & Schulz, 1962) and of deoxycoformycin (Camisa, Grever & Bouroncle, 1985).

Which cytotoxic drug or combination of drugs caused the reaction in our patient is not clear. However, it is of interest that both doxorubicin and 5-fluorouracil cause radiation recall reactions (Hood & Haynes, 1983). As actinic keratoses are caused by long-term damage to the skin by UV radiation, their inflammation may be considered a form of radiation recall reaction. It is possible that the dysplastic cells of actinic keratoses bear accumulated UV-induced damage in their DNA which renders them more susceptible to the toxic effects of cytotoxic drugs.

Clearly this reaction is different from the development of solar keratoses and cutaneous malignancies in chronically immunosuppressed transplant recipients. In these cases the lesions appear to develop as a result of impaired immunosurveillance, and the clinical course is different.

The recognition of this reaction is of clinical importance as it may be misdiagnosed as a drug allergy, resulting in the unnecessary withholding of drugs. The most important clue to the true nature of the eruption is its restriction to sun-damaged skin.

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## Dapsone therapy for oral-genital ulceration

MADAM, We feel it necessary to comment on the correspondence from Handfield-Jones, Allen and Littlewood (*British Journal of Dermatology* 1985, **113**, 501) describing treatment of oral-genital ulceration (OGU) and oral ulceration alone with a combination regime of dapsone, zinc sulphate and co-trimoxazole.

Sixteen of their patients had either oral ulceration or OGU unassociated with any other disease. Whether these ulcers were subjected to histological or immunofluorescent studies is not stated. It is our experience that these forms of ulceration may be the sole presenting symptom of linear IgA disease (LAD) or benign mucous membrane pemphigoid (BMMP), ocular lesions being discovered only on ophthalmological assessment performed by an expert in these disorders (Leonard *et al.*, 1984).

The diagnosis of both LAD and BMMP can be made on direct immunofluorescence of peri-lesional mucous membrane, and in the case of LAD from uninvolved skin, the presence of a homogeneous linear band of IgA, C3, along the basement membrane being characteristic.

Rogers, Seehafer and Perry (1982) described the therapeutic effect of dapsone on the oral ulceration of BMMP and we have found that the sulphonamides, sulphamethoxypyridazine and sulphapyridine are equally beneficial.

It is possible that a number of the 19 patients reported by Handfield-Jones *et al.* were in fact suffering from either BMMP or LAD and therefore would have responded to either sulphapyridine or sulphamethoxypyridazine. As a general rule in our dermatitis herpetiformis patients, we do not prescribe dapsone over the age of 50. Haemolysis is a very common side effect with this drug, and a significant fall in haemoglobin in patients over 50 may well precipitate cardiac failure or angina, even progressing to myocardial infarction. It is significant that in 4 of the 19 patients reported, there was indeed a marked fall in haemoglobin. Since BMMP is more common in patients over 50, we feel that dapsone should not be included as a first line drug.

As Handfield-Jones *et al.* admit, their regime for OGU was developed empirically. Although empiricisim is all too common in medicine, and in dermatology in particular, advances have been made, and there are now suitable investigations to enable us to make a definitive diagnosis in a large proportion of patients with OGU. If they have either LAD or BMMP their oral and genital ulcers may well respond to treatment with sulphapyridine or sulphamethoxypyridazine, and the incidence of side effects will be considerably less than with the triple drug combination of dapsone, zinc sulphate and co-trimoxazole.

Dermatology Department,
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C.E.M.GRIFFITHS
J.N.LEONARD
L.FRY

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LEONARD, J.N., WRIGHT, P., WILLIAMS, D.M., GILKES, J.J.H., HAFFENDEN, G.P., MCMINN, R.M.H. & FRY, L. (1984) The relationship between linear IgA disease and benign mucous membrane pemphigoid. *British Journal of Dermatology*, **110**, 307.

ROGERS, R.S., SEEHAFER, J.R. & PERRY, H.O. (1982) Treatment of cicatricial (benign mucous membrane) pemphigoid with dapsone. *Journal of the American Academy of Dermatology*, **6**, 215.

## Reply

MADAM, We agree with Dr Griffiths and his colleagues that LAD can present with oral ulceration alone and have described this previously (Allen & Reeves, 1983) but none of our patients had evidence of this condition.

BMMP should not normally cause problems in diagnosis because, apart from differences in appearance and distribution, the individual ulcers tend to be chronic rather than recurrent, as is the case with the severe aphthosis type to which we were referring. Nevertheless, we agree that linear IgA deposition may be an occasional feature of BMMP (Maurice *et al.*, 1986).

Empiricism is common in medicine; that is how the effect of dapsone itself was first discovered, but the main purpose of our letter was to indicate that the combination therapy gives additional benefits in comparison with this drug alone. We have not tried sulphapyridine in place of dapsone.

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University Hospital,
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S.HANDFIELD-JONES
B.R.ALLEN
S.M.LITTLEWOOD

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ALLEN, B.R. & REEVES, W.G. (1983) Skin diseases. In: *Immunology in Medicine*, (Ed. by E.J. Holborow and W.G. Reeves) 2nd edn. p. 421. Grune and Stratton, London.

MAURICE, P.D.L., ALLEN, B.R., MARRIOTT, D.W., POWELL, R.J. & REEVES, W.G. (1986) Skin Immunofluorescence in the diagnosis of primary bullous disease. *Clinical and Experimental Dermatology*. (in press)

## Delusions of cutaneous wire wool

MADAM, I recently saw a patient who provided a new variation on the theme of delusions of parasitosis (Lyell, 1983). A 47-year-old matron of a nursing home was referred by her general practitioner with the diagnosis of hand eczema which had failed to respond to treatment. The patient said that about 10 years previously she had once used a wire wool brush to clean a sink, when small particles of wire had become embedded in her fingers. These caused no trouble for several years, but she gradually became more and more aware of their presence and for the last 6 months she had had to pick out particles of metal from her skin every day in order to 'prevent sepsis'.

On examination, the fingers of both hands were covered in crusted, gouged-out excoriations, due to picking. X-ray of the hands was normal. The 'metal' specimens which the patient proudly produced in the traditional matchbox consisted of particles of dried skin, with no metal present on microscopic or chemical examination.

The patient appeared mentally and physically normal apart from this monosymptomatic delusional state. Treatment with pimozide produced no improvement. The delusion was shared by her daughter, a research chemist, who suggested that the particles were obviously too small to be seen or identified by conventional methods, and she felt that investigation by nuclear magnetic resonance was indicated.

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Bristol Royal Infirmary,
Bristol BS2 8HW, U.K.

J.L.BURTON

REFERENCE

LYELL, A. (1983) Delusions of parasitosis. *British Journal of Dermatology*, **108**, 485.

## Light sensitivity versus mycosis fungoides

MADAM, We sympathize fully with the problems experienced by Neill and du Vivier (*British Journal of Dermatology* 1985, **113**, 497) in differentiating mycosis fungoides from actinic reticuloid. Indeed, we have no reason to doubt the accuracy of their final diagnosis of mycosis fungoides. However, we note that although the published photomicrograph shows cellular epidermotrophism, more reliable histological criteria of mycosis fungoides (such as Pautrier abscesses and vacuolar interface dermatitis) were neither illustrated nor stated to be present. In addition, more definitive modern methods of diagnosing mycosis fungoides, such as monoclonal antibodies against tumour associated antigens and clonal rearrangements of beta T cell receptor gene DNA, were understandably not utilized.

We, ourselves, have found that the histological distinction between light sensitivity and mycotic eruptions is a difficult and not infrequent problem. However, we believe that T4:T8 lymphocyte ratios within the tissue may be of value in this distinction. As is now widely recognized, and present in the case described by Neill and du Vivier, mycosis fungoides is invariably characterized by a predominant proliferation of T4 cells. In contrast, all light sensitivity eruptions studied to date by ourselves (including actinic reticuloid) show a predominant T8 reactivity.

We wonder whether other research groups find a low T4:T8 ratio with T8 excess to be a valuable means of differentiating this histological problem?

Pathology Department,
University of Sheffield Medical School,
Sheffield S10 2RX, U.K.

D. Slater
N. Rooney

*British Journal of Dermatology* (1986) **114**, 644.

# *News and Notices*

## Conference on Bullous Dermatoses

A conference on bullous dermatoses will be held in Zagreb from 9–11 October 1986. Details and a copy of the preliminary programme may be obtained from Professor Dr Alaksej Kansky, Klinika Bolnickog Centra, Salata 4, Zagreb, Yugoslavia.

## 5th Conference of the European Society for Comparative Skin Biology

The 5th Conference of the European Society for Comparative Skin Biology will be held at the J.W.Goethe University in Frankfurt from 1–5 September 1986, and will include oral communications, invited lectures, posters and workshops. All aspects of skin biology will be covered, with special emphasis on differentiation, wound healing, and the skin as a sensory organ. For further details please contact Professor J. Bereiter-Hahn, Kinem Zell Forschung der J.W.Goethe Universitet Frankfurt, Senckenberganlage 27, D-6000, Frankfurt a M., Federal Republic of Germany.

## International Congress on the Biological and Clinical Aspects of Phagocyte Function

The above congress is to be held at the University of Pavia, Italy from 7–10 September 1986. Abstracts should be submitted before 31 May 1986. Further information is available from Dr G.Ricevuti, Istituto di Patologia Speciale Medica $1^a$, Università di Pavia, 27100 Pavia, Italy.

## An Activity Sponsored by the Basotherm Förderkreis e.V. Theodor Nasemann Grant—Dermatovirology

The grant comprises DM 20 000 and a 3 month research scholarship aimed at giving young scientists the opportunity to become familiar with new investigation methods and to promote studies in the field of dermatology-virology by means of laboratory testing.

The Theodor Nasemann grant created by the Basotherm Förderkreis e.V. and Basoderm A/S, in collaboration with the director of the Hamburg University Clinic of Dermatology, is awarded every 2 years.

The grant and scholarship is open to scientists under 40 years of age from Belgium, the Federal Republic of Germany and West Berlin, Denmark, Finland, Great Britain, Italy, the Netherlands, Luxembourg, Norway, Austria, Portugal, Sweden, Switzerland and Spain, who are able to present a study in dermatovirology deemed to be of sufficient merit.

Applications for this scholarship and grant should be sponsored by the head of the relevant dermatology department and sent in by 1 June 1986, at the latest, to Prof. Dr. Med. Theodor Nasemann, Direktor der Univ. Hautklinik, Martinistrasse 52, D-2000 Hamburg 20, Federal Republic of Germany.

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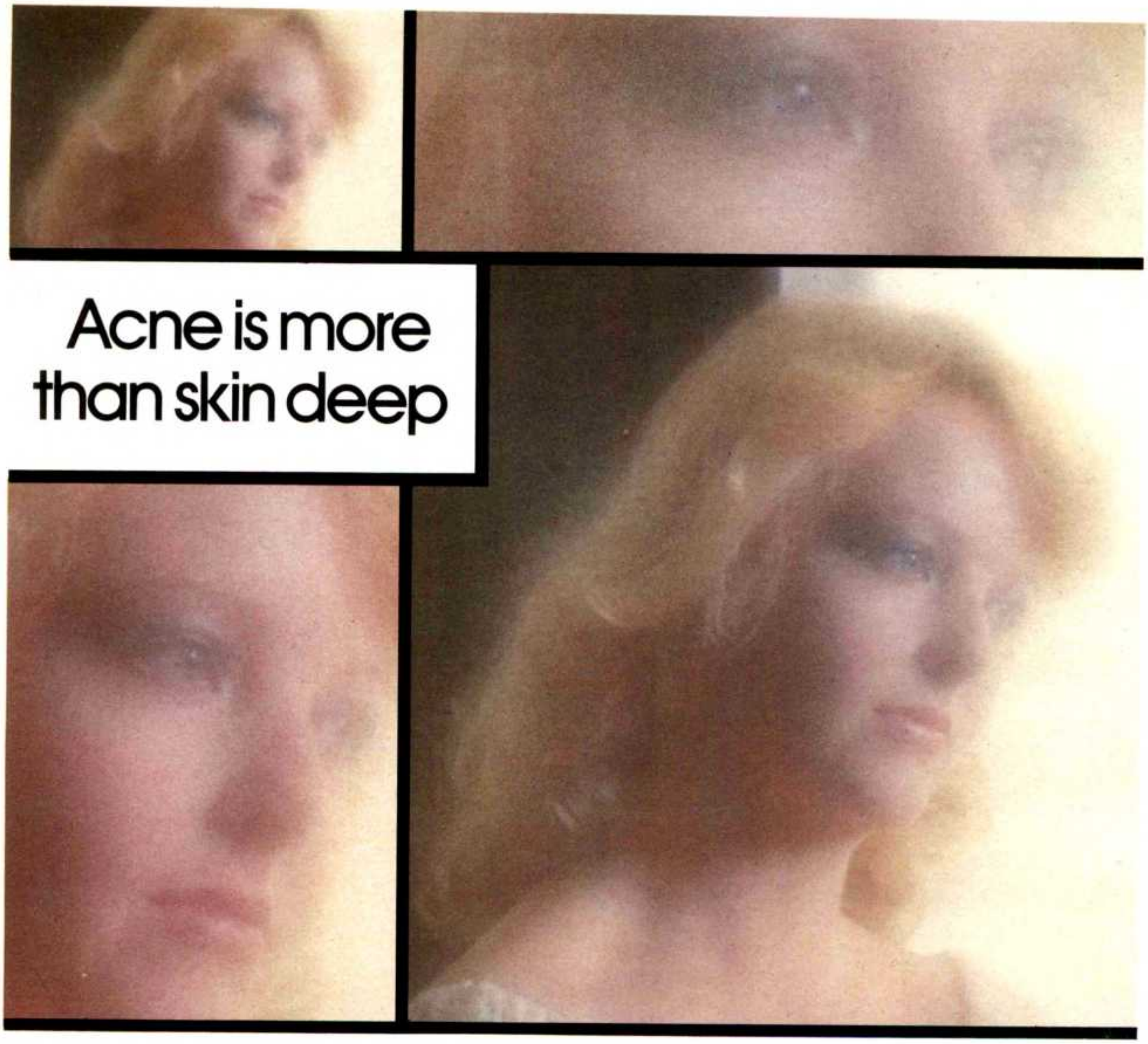
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| kilogram(s) | kg | centimetre(s) | cm | millilitre(s) | ml |
| milligram(s) ($10^{-3}$g) | mg | second(s) | s | gravitational acceleration | *g* |
| microgram(s) ($10^{-6}$g) | μg | cubic millimetre(s) | mm$^3$ | micrometre(s) | μm |
| nanogram(s) ($10^{-9}$g) | ng | millimetre(s) | mm | per cent | % |
| picogram(s) ($10^{-12}$g) | pg | millicurie(s) | mCi | isotopic mass number placed as | $^{131}$I |
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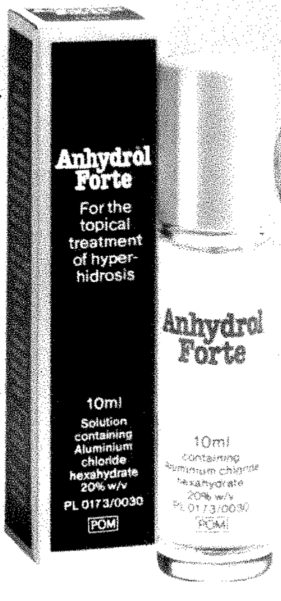
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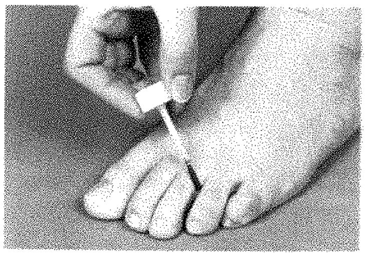

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1 Krebs HA, Wiggins D, Stubbs M, Sols A, Bedoya F. Studies on the mechanism of the anti-fungal action of benzoate. Biochem J 1983, **214**: 657-63

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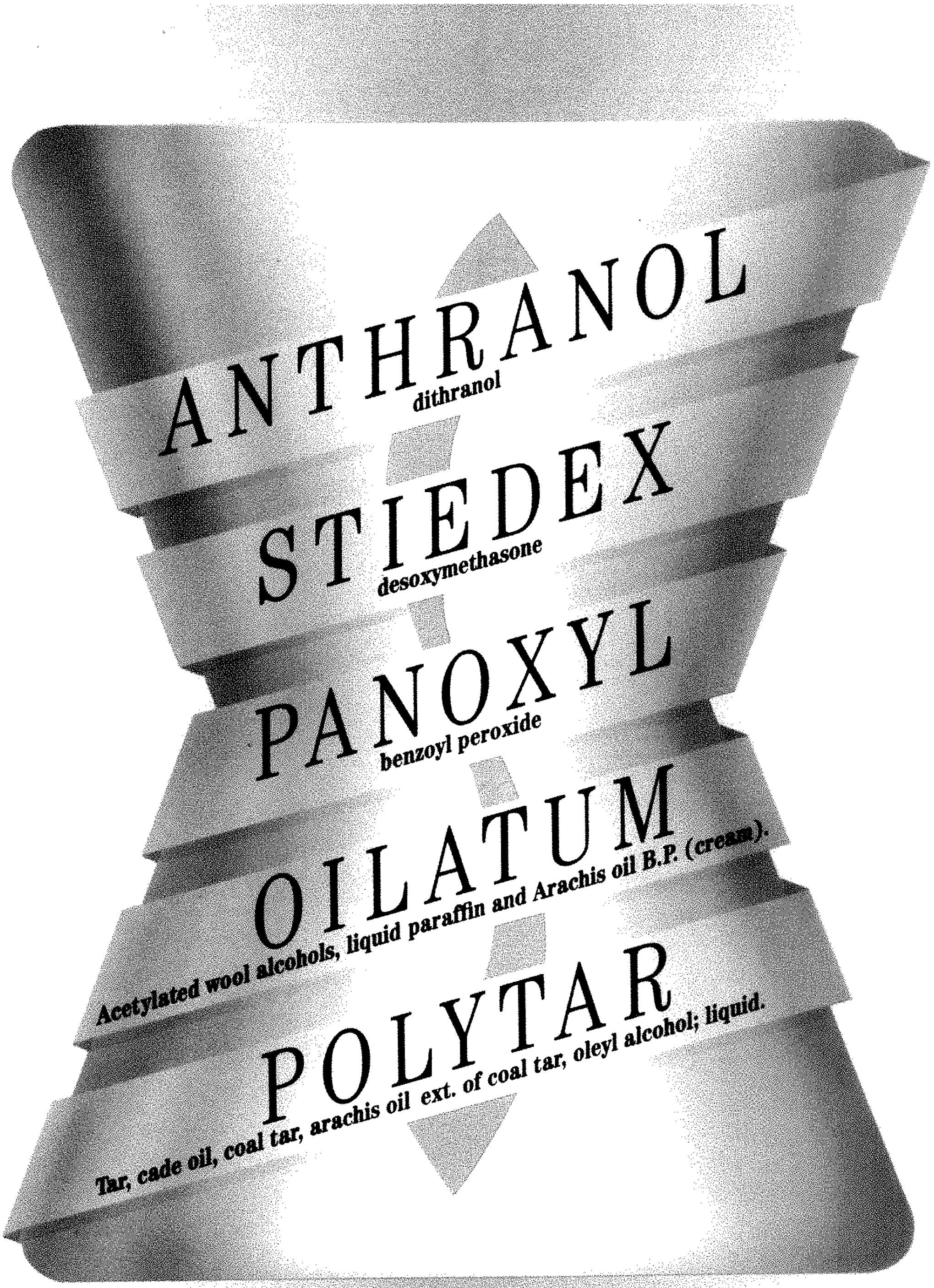

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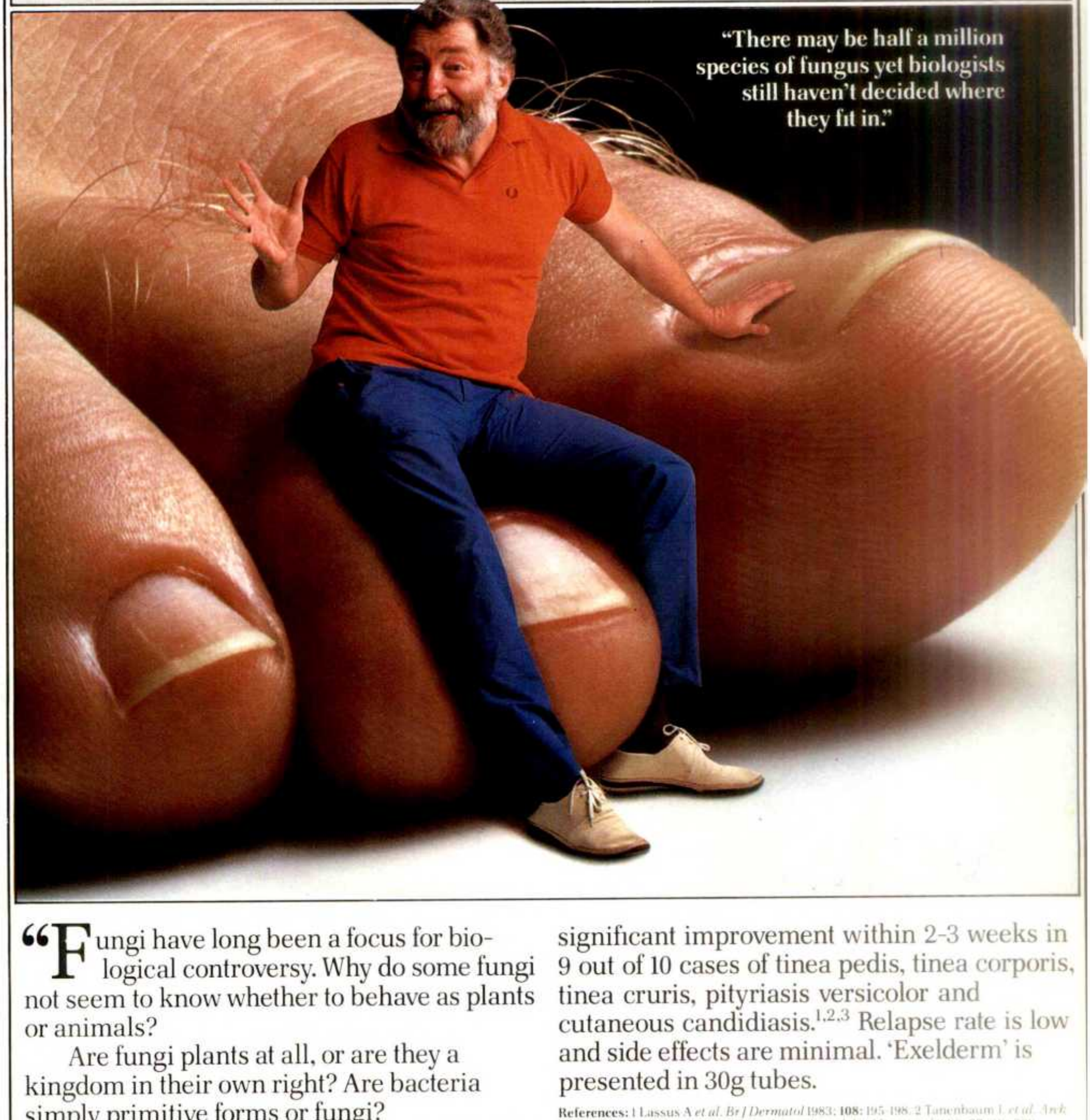
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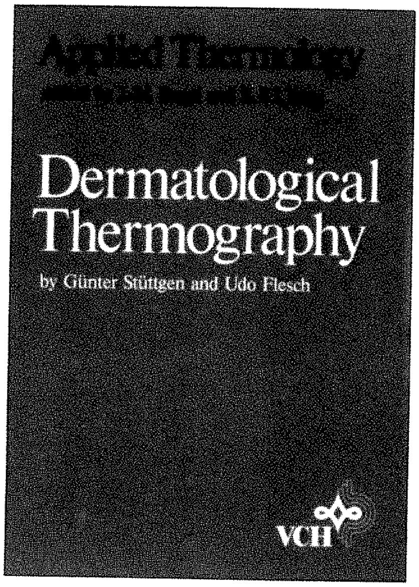
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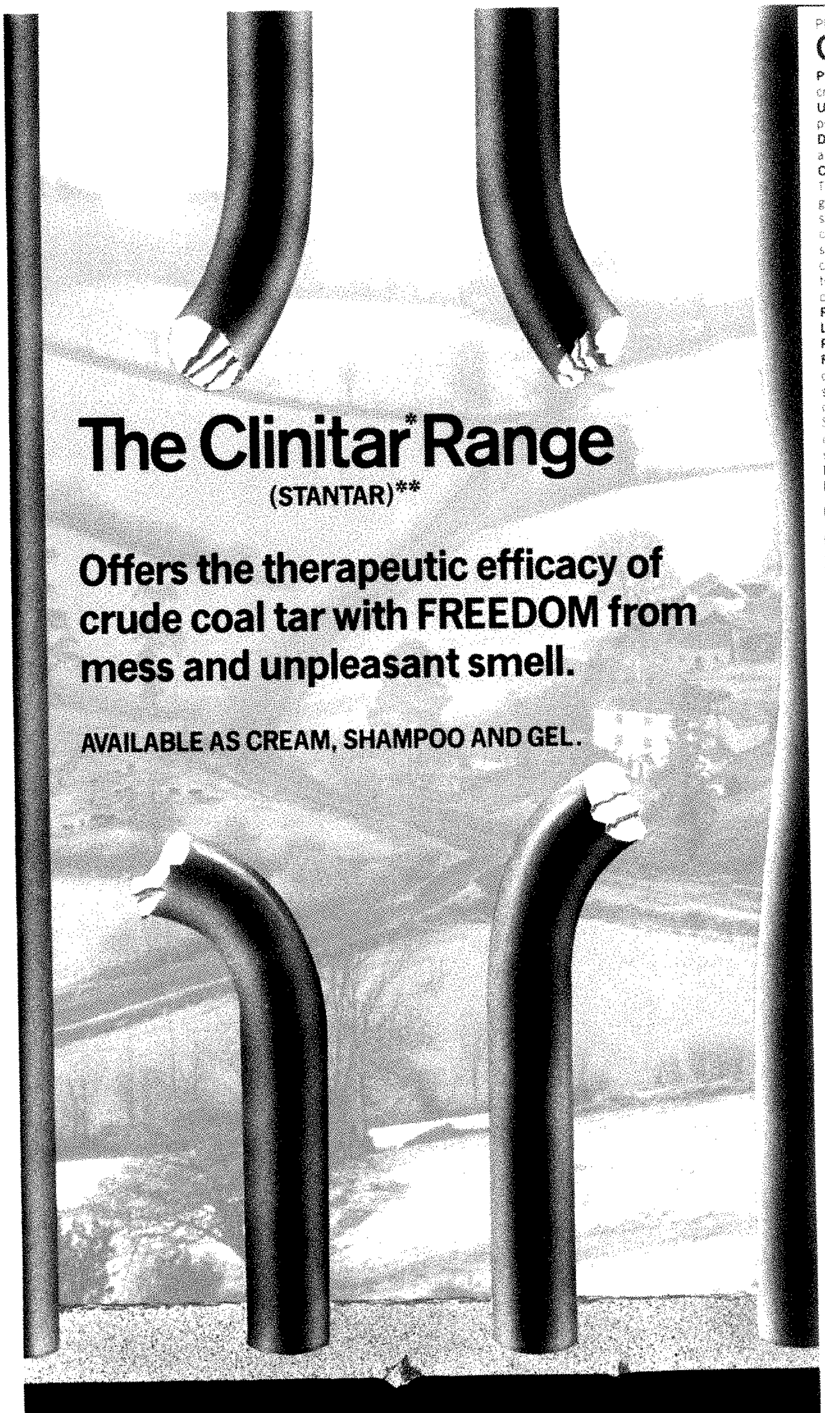
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*British Journal of Dermatology* (1986) **114**, 645–650.

***Comment***

# Expectations of human epidermal kinetic homeostasis

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In 1980, Marks, in this journal, commented critically on current views on mammalian epidermal homeostasis. Although interest in toad skin may appear strange to a dermatologist, some of the suggestions and questions raised by Marks have been analysed on toad epidermis, which constitutes a unique model system for the study of tissue homeostatic mechanisms (Budtz, 1982, 1985a). The purpose of this article is to compare the knowledge gained from such studies with some of the available information necessary to assess human epidermal homeostatic mechanisms.

It has been shown repeatedly that the toad epidermal cell pool size is fairly constant (Budtz, 1979, 1982, 1985a,b), which—as in any non-expanding epidermis—may be considered to reflect a balance between the number of cells lost by desquamation and a corresponding replacement of these cells by divisions in the basal epidermis. In other words, the desquamation rate equals the mitotic rate (Bergstresser & Taylor, 1977; Budtz, 1979; Weinstein, McCullough & Ross, 1984; Hume, 1985). For the epidermis of toads, however, it has been shown recently, that this concept does not necessarily hold; the cell birth rate in intact toads exceeds the rate of cell loss through moulting by about 170%. It has been argued that this 'surplus' of cells could not be permanently accommodated within the living epidermis, and hence it was concluded that the 'surplus' of cells is deleted by an as yet undefined process (possibly apoptosis) (Budtz, 1985a). It was further suggested that this deletion takes place in an apparently controlled manner (Budtz, 1985b). In toads deprived of their pars distalis of the pituitary gland, an operation which leads to an increased rate of formation of corneal cells (Larsen, 1976; Budtz, 1977), the change in the epidermal cell pool size was not compatible with cell deletion exceeding corneal cell formation by about 170%. Evidently, following the operation, all cells produced were now used for the excessive formation of corneal cells, thus lending support to the hypothesis that controlled cell deletion may be a tissue homeostatic mechanism complementary to controlled cell division (Kerr, Wyllie & Currie, 1972; Marks, 1980; Wyllie, 1981).

Which of the two above concepts applies to human skin? In spite of its vital importance to our understanding of normal and diseased human skin we must conclude that at the present time we do not really know; arguments for this view are presented below.

### *Ways of expressing epidermal cell pool size, cell birth rate and desquamation rate*

Most often these parameters are expressed as cells per 1000 basal cells (or per mm) per hour (or

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per day). However, in the context of epidermal homeostasis it is important to recognize the three-dimensional nature of the epidermis. Hence the cell birth rate ($k_B$) and the desquamation rate ($k_D$), as well as the size of the epidermal cell pool, should be expressed not per unit length of surface, but per unit area of surface. It has been demonstrated that expressing these parameters per unit length of surface may indicate perfect matching between cell loss and cell production ('steady state compartment'), although calculating from the surface length data the values of these parameters per unit area of skin clearly shows that the compartment is expanding, the $k_B$ being much higher than the $k_D$ (Budtz, 1985a). Conclusions drawn from surface length relationships between $k_B$ and $k_D$ may therefore be totally misleading.

Since one of the few serious attempts at analysing $k_B$, $k_D$ and cell pool size expressed per unit area of skin is presented in a recent paper by Weinstein *et al.* (1984), data drawn mainly from their paper will be used to illustrate some of the problems encountered in assessing human epidermal homeostatic mechanisms. According to these authors, there is fairly good agreement between the daily cell birth rate (as calculated from their experimental data) and the daily desquamation rate (as calculated from data in the literature) and thus they claim that: 'the expected kinetic homeostasis is confirmed'. Evidently, this statement depends on expectations, since at least two mechanisms for maintenance of a constant epidermal cell pool size may be tested against each other, one claiming rates of mitosis and desquamation as the sole determinants of epidermal cell pool size, the other claiming controlled cell deletion (beyond that of desquamation) as an additional epidermal homeostatic mechanism.

### *The cell birth rate*

The $k_B$ is principally measured by two methods: indirectly by means of $^3$H-thymidine incorporation into DNA during the S-phase, and directly by means of metaphase-blocking agents and measurement of the metaphase arrest (Wright & Alison, 1984). Considering the ethics of using these agents in healthy humans, it is not surprising that there are relatively few publications on human $k_B$. Weinstein *et al.* (1984) calculated a cell cycle duration ($T_c$) of 311 h from the equation

$$T_c = \frac{T_s \times GF}{LI}, \tag{1}$$

where the duration of the S-phase ($T_s$) was estimated to be 14·0 h by the flash-labelled mitosis method (measured at the 37% points), and a growth fraction ($GF$) of 60% was assumed on the basis of the literature ($LI$ = labelling index). Application of equation 1 presupposes, however, a rectangular age distribution within the epidermis, an assumption which has still to be demonstrated. Further, these authors found an average $GF$ of 89·3% for human skin implants. A $k_B$ of 1246 cells/mm$^2$/day was calculated according to the equation

$$k_B = \frac{GF}{T_c}, \tag{2}$$

where $GF$ is the number of proliferating cells (60% of 27000) and $T_c$ = 311 h. However, depending on the choice of $GF$ and age distribution, different values of $k_B$ could be calculated (Table 1). The data in the paper by Weinstein *et al.* (1984) also may be used to recalculate the $k_B$ in a different way (Fig. 1). The resulting values for $k_B$, which are about twice those calculated by these authors, are also included in Table 1.

TABLE 1. $k_B/k_D$ ratios calculated using various assumptions*

| Cell birth rates ($k_B$) | Desquamation rates ($k_D$) 1470 (g) | 658 (h) | 1370 (j) | 619 (k) |
|---|---|---|---|---|
| 1246 (a) | 0·84 | 1·89 | 0·98 | 2·01 |
| 1875 (b) | 1·26 | 2·85 | 1·48 | 3·03 |
| 976 (c) | 0·66 | 1·48 | 0·77 | 1·58 |
| 1337 (d) | 0·92 | 2·09 | 1·08 | 2·22 |
| 2363 (e) | 1·59 | 3·59 | 1·86 | 3·82 |
| 2645 (f) | 1·78 | 4·02 | 2·08 | 4·27 |

* Assumptions and references:

(a)–(f): calculations from data in Weinstein *et al.* (1984);

(a) age distribution rectangular, *GF* 60% (Weinstein *et al.*, 1984);

(b) age distribution rectangular, *GF* 90%;

(c) age distribution exponential, *GF* 60% (eq. 2·13 in Wright & Alison, 1984);

(d) age distribution exponential, *GF* 90% (eq. 2·13 in Wright & Alison, 1984);

(e) and (f) calculations (*see* Fig. 1);

(g) Weinstein *et al.* (1984);

(h) abdomen (Table 2);

(j) lower arm (Table 2);

(k) thigh (Table 2).

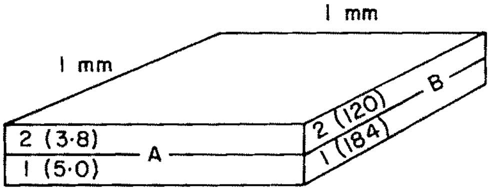

FIGURE 1. Recalculation of human epidermal cell birth ($k_B$) from data presented by Weinstein *et al.* (1984). The diagram illustrates the basal (row 1) and suprabasal (row 2) layers of the proliferative compartment in 1 mm² of epidermis. From the data given, expressed as labelled cells per 100 basal cells, and from data on number of cells per mm in row 1 and in row 1 + 2 respectively, the number of labelled cells in rows 1 and 2 can be calculated, and are shown in brackets in panel A. With a $T_S$ of 14 h, and assuming a rectangular age distribution (see text), the

$$k_B = \frac{LI}{T_S}$$

may be calculated to be 0·335 cells/mm/h and 0·276 cells/mm/h for rows 1 and 2 respectively. In panel B the numbers of cells per mm in rows 1 and 2 are shown in parentheses. In row 1 there are 184 cells/mm, corresponding to 184 rows of cells, each producing 0·335 cells/mm/h. Hence a $k_B$ of 0·335 × 184, i.e. 65·32 cells/mm²/h can be calculated. Similarly, $k_B$ of row 2 can be calculated to be 33·12 cells mm²/h, and thus the total $k_B$ is 98·14 cells/mm²/h or 2363 cells/mm²/day. With the shorter estimate of $T_S$ of 12·5 h, indicated by the computer analysis of the FLM data, a $k_B$ of 2645 cells/mm²/day can be calculated.

*Corneal compartment and cell loss by desquamation*

The daily rate of desquamation per mm$^2$ of surface ($k_D$) may be calculated according to the equation

$$k_D = \frac{N_c \times N_L}{T}, \tag{3}$$

where $N_c$ = the number of cells per mm$^2$ in one corneal layer, $N_L$ = the number of layers within the corneal compartment, and $T$ = the corneal transit time. All three parameters vary with the body site (Table 2). Thus, the corneocyte area varies from 750 to 1250 $\mu$m$^2$ (Plewig & Marples, 1970), and hence the number of corneocytes in one corneal layer ranges from 800 to 1300 cells/mm$^2$. If these figures are correct, it is interesting to note that by the scrubbing technique no more than the outermost or two outermost layers are removed. Thus, Roberts & Marks (1980) could, by 'forced desquamation', release 556–1040 cells/mm$^2$ and McGinley, Marples & Plewig (1969) 618–2280 cells/mm$^2$ from various body sites, which represents 10% or less of the total corneal compartment (Table 2). The number of corneal layers varies from 17 to 22 (Holbrook & Odland, 1974), and by measuring the disappearance of dansyl chloride fluorescence, the corneal transit time has been estimated to vary from 18·5 to 26·5 days (Roberts & Marks, 1980). On the basis of these data, desquamation rates ranging from 620 to 1270 cells/mm$^2$/day may be calculated for various body sites (Table 2). The 'passive desquamation rates' of 156–314 cells/mm$^2$/day (Roberts & Marks, 1980) are probably minimum values.

TABLE 2. Human epidermal desquamation rates

| | Abdomen | Lower arm | Thigh | Back |
|---|---|---|---|---|
| Corneocyte area ($\mu$m$^2$) (a) | 1056 | 937 | 1158 | |
| Corneocytes per mm$^2$ in one corneal layer (b) | 947 | 1067 | 864 | |
| Number of corneal layers (c) | 18 | 22 | 19 | 17 |
| Corneal compartment (total number of corneocytes per mm$^2$ of surface) (d) | 17046 | 23479 | 16416 | |
| Corneal transit time (days) (e) | 25·9 | 18·5 | 26·5 | 23·6 |
| Calculated desquamation rate (cells per mm$^2$ per day) (f) | 658 | 1270 | 619 | |

References:
(a) Plewig (1970);
(b) calculated from (a);
(c) Holbrook & Odland (1974);
(d) calculated from (b) and (c);
(e) Roberts & Marks (1980);
(f) calculated from (d) and (e) (Equation 3).

*Epidermal tissue kinetic homeostasis*

From the above considerations it appears obvious that in the context of epidermal homeostasis, it is of paramount importance to compare cell birth rate with desquamation rate from the same body site. Moreover, sex and age should be taken into consideration (Plewig, 1970). Values of $k_B$

calculated using various assumptions are shown in Table 1, in which values for $k_D$ are also shown. For the various combinations of $k_B$ and $k_D$, the ratio $k_B/k_D$ has been calculated and, as can be seen, any ratio between 0·66 and 4·27 can be arrived at. If the 'non sun-exposed skin' of Weinstein *et al.* (1984) was taken from the abdomen or the thigh, these ratios indicate, independently of the choice of assumption, a cell production rate in excess of the rate of cell loss by desquamation.

### *Perspectives and conclusion*

It should be emphasized that nothing is proven by the above calculations since no experimental data have been published on human epidermal cell birth rate and desquamation rate measured in the same individuals at the same body site and at the same time. Thus, at present it is impossible to say whether, in human epidermis, mitotic and desquamation rates are the only parameters determining the size of the epidermal cell pool, or whether the concept of controlled cell deletion as an additional tissue homeostatic mechanism could apply also to human epidermis. The findings on toad epidermis (Budtz, 1985a, 1985b), and the above calculations, suggest that the latter possibility should be seriously investigated. If it is so, an intriguing question would be: What is the physiological significance of such an 'expensive' mechanism? As pointed out by Marks (1980), epidermal mitotic activity is influenced by a number of hormones and other agents which may have primary functions other than controlling mitotic activity. Stimulation or inhibition of mitotic activity by changes in one of these substances, or the concerted influence of several, may be counterbalanced by changes in the rate of cell deletion (over and above desquamation) and thus prevent inappropriate variation in epidermal cell population size. Support for this interpretation comes from pars distalisectomized toads, which showed an increased rate of desquamation and a decreased mitotic rate, and yet a much smaller decrease in epidermal population size than expected; an observation which was explicable by assuming abolition of cell deletion (over and above desquamation) (Budtz, 1985b).

As has been pointed out recently by Hume (1985), keratinocytes are 'at risk' from a range of aetiological agents, and proliferative hierarchies conferring protective mechanisms in surface epithelia were discussed. An expected spontaneous mutation rate of $10^{-6}$ genes/cell division might produce observable phenotypic changes, including neoplasia, in the epithelium as the animal ages, but such changes are seen only rarely. Thus, the production of a 'surplus' of cells, as in toads, would provide the epidermis with a pool of newly divided cells from which the 'best fitted', i.e. non-mutated/non-damaged, cells could be selected for transit and eventual desquamation, while the remaining, non-stem cells were deleted in some way. Interestingly, in toads deprived of the pars distalis, there is a correlation between abnormal keratinization (Budtz, 1979) and the abolition of cell deletion (Budtz, 1985). Whether there is a causal relationship between the two phenomena, however, remains to be demonstrated.

If the concept of controlled cell deletion as a tissue homeostatic mechanism additional to controlled cell division applies also to human epidermis, another important question is: What is the fate of the 'surplus' cells? If deleted by apoptosis (Kerr *et al.*, 1972; Marks, 1980; Wyllie, 1981), the incidence of apoptotic cells in histological sections would be small; calculated for toads to be 0·06 cells/mm/h (Budtz, 1985a). Thus, the frequency by which apoptotic cells would be seen depends upon the duration of the apoptotic process. However, as in all circumstances investigated, this appears to be short (Wyllie, 1981); it is not surprising that apoptotic cells are seen only rarely. In this context it is worth noting that in psoriasis, in which the mitotic activity is increased, a large number of apoptotic cells can be seen (Olson & Everett, 1975; Wright, 1981).

It is hoped that the analysis of human epidermal kinetic data presented here, seen in relation to the revised concept of homeostatic mechanisms in a lower vertebrate, will stimulate further research on human epidermal homeostatic mechanism(s).

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***Clinical and Laboratory Investigations***

# Anatomical distribution and immunological characteristics of epidermolysis bullosa acquisita antigen and bullous pemphigoid antigen

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## SUMMARY

A Japanese patient with epidermolysis bullosa acquisita (EBA) was autopsied, and direct immunofluorescence (DIF) testing was performed. Using this patient's serum (EBA serum) and three bullous pemphigoid (BP) sera, the anatomical distribution and immunological characteristics of EBA antigen and BP antigen were investigated by indirect immunofluorescence (IIF). EBA antigen showed the same anatomical distribution as BP antigen in DIF and IIF studies; both antigens were limited to the skin, tongue, oesophagus, trachea, cornea and bladder. EBA antigen was located on the dermal side of both NaCl and PBS-separated skin, whereas BP antigen was limited to the epidermal side. Ethanol fixation abrogated the antigenic stability of BP antigen, but not that of EBA antigen. No difference was found when acetone or formalin fixation was used. The separation methods and prefixation in ethanol could be useful techniques applicable to the classification of the bullous disorders which manifest circulating anti-BMZ antibodies.

Epidermolysis bullosa acquisita (EBA) is a rare non-hereditary mechanobullous disorder defined by (i) trauma-induced bullae occurring over the joints of the hands, feet, elbows, and knees; atrophic scars, milia, and nail dystrophy; (ii) post-infancy onset; (iii) no family history of EBA; (iv) exclusion of other bullous diseases; (v) demonstration of IgG in the basement membrane zone (BMZ) by direct immunofluorescence microscopy; (vi) demonstration of blister formation beneath the basal lamina, remaining on the epidermal side of the blister, by electron microscopy, and (vii) deposits of IgG in the amorphous zone beneath the basal lamina, demonstrable by immunoelectron microscopy (Roenick & Pearson, 1981).

The critical difference between EBA and bullous pemphigoid (BP), both of which show linear deposits of immunoglobulin and complement along the BMZ, lies in the immunoelectron microscopic findings. In EBA the immune deposits are located in the upper dermis beneath the

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basal lamina (Nieboer *et al.*, 1980; Yaoita *et al.*, 1981; Gammon, Briggaman & Wheeler, 1982; Matsukawa *et al.*, 1983), whereas in BP they are located in the lamina lucida (Holubar *et al.*, 1975).

Recently, Woodley *et al.* (1984) have identified a novel epidermal antigen (EBA antigen) using the sera of EBA patients, proving that EBA is a distinct entity.

In the present study, we used direct immunofluorescence to determine the distribution of EBA antigen in organs obtained at autopsy from a patient with EBA. The distribution of EBA antigen in the organs of several other mammals and a control human cadaver was investigated using an indirect immunofluorescence procedure with the serum of the EBA patient. The immunological characteristics of EBA antigen were investigated and compared with those of BP antigen.

## METHODS

### *Tissue samples*

Tissue samples were obtained at autopsy from a 60-year-old Japanese man who had been suffering from EBA. This patient was the first reported case of EBA in Japan.

Clinical, histological, ultrastructural, immunofluorescence, and immunoelectron microscopic findings have been reported elsewhere (Matsukawa *et al.*, 1983; Furue *et al.*, 1986). Tissue samples were obtained from various organs, and snap frozen with O.C.T. compound (Miles Laboratories, Naperville, Illinois, U.S.A.) in liquid nitrogen and processed for direct immunofluorescence (DIF) studies. Tissue samples from various organs were also obtained from mice, guinea-pigs, rabbits and a non-affected human cadaver.

For skin separation and pre-treatment with fixatives, samples of normal human skin were obtained from five patients at surgery. Each sample was divided in half. One half was used for separation of the skin at dermal-epidermal junction (DEJ). The other was kept at −70°C in O.C.T. compound and used for experiments with different fixatives.

### *Sera*

Sera were obtained from the patient with EBA (EBA serum) and from three patients with BP. Each of these sera was shown to contain IgG class anti-BMZ antibody using normal human skin as substrate. The titres of antibodies were as follows: EBA, 1:320; BP 1:640, 1:160 and 1:640 respectively. Sera from two normal subjects were used as controls.

### *Antibodies*

Fluorescein isothiocyanate (FITC) conjugated goat anti-human IgG [$F(ab)_2$ specific], peroxidase conjugated goat anti-human IgG [$F(ab)_2$ specific] and FITC conjugated goat anti-human C3 were all purchased from Cappel Laboratories (Cochranville, PA, U.S.A.).

### *Direct immunofluorescence*

Direct immunofluorescence was performed for IgG and C3 as described previously (Matsukawa *et al.*, 1983). FITC conjugated goat anti-human IgG or C3 were used at a dilution of 1:160.

### *Indirect immunofluorescence*

Indirect immunofluorescence (IIF) was performed as described previously (Matsukawa *et al.*, 1983). EBA and BP sera were used at a dilution of 1:10. FITC conjugated goat anti-human IgG was used at a dilution of 1:160.

### *Indirect immunoelectron microscopy*

In order to localize precisely the ultrastructural binding sites, indirect immunoelectron microscopy (IIEM) was performed with EBA serum and three BP sera using a modification of the method of Chu, Bhogal and Black (1983). Briefly, 6 $\mu$m sections of normal human skin were incubated with each serum sample (diluted, 1:20). After washing in PBS, the sections were incubated with peroxidase conjugated goat anti-human IgG (diluted, 1:80). After three washes, they were fixed in 2·5% glutaraldehyde for 30 min, then immersed in 0·1 M Tris buffer containing diaminobenzidine and hydrogen peroxide. After rinsing, the sections were postfixed in 2% $OsO_4$ and dehydrated in a series of alcohols. Finally, they were embedded in Epon 812 for 72 h at 60°C. Ultrathin sections were cut and were observed with a JEM-100C electron microscope operating at 80 kV.

### *Separation of human skin at the dermal-epidermal junction*

A 0·5 cm piece was cut from each skin sample and the subcutaneous fat scraped off. Two

TABLE 1. Organ and species specificity of EBA and BP sera

| | Direct immunofluorescence | Indirect immunofluorescence | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Patient | Mouse | | Guinea-pig | | Rabbit | | Human* | |
| | EBA | EBA | BP | EBA | BP | EBA | BP | EBA | BP |
| Skin | + | + | + | + | + | + | + | + | + |
| Tongue | + | + | + | + | + | + | + | + | + |
| Upper-oesophagus | + | + | + | + | + | + | + | + | + |
| Lower oesophagus | + | + | + | + | + | + | + | + | + |
| Stomach | – | – | – | – | – | – | – | – | – |
| Duodenum | – | – | – | – | – | – | – | – | – |
| Jejunum | – | – | – | – | – | – | – | – | – |
| Colon | – | – | – | – | – | – | – | – | – |
| Pancreas | – | ND | ND | ND | ND | ND | ND | – | – |
| Liver | – | – | – | – | – | – | – | – | – |
| Kidney | +** | – | – | – | – | – | – | – | – |
| Lung | – | – | – | – | – | – | – | – | – |
| Bladder | ND† | ND | ND | (+)‡ | (+) | (+) | (+) | (+) | (+) |
| Ureter | – | ND | ND | ND | ND | ND | ND | – | – |
| Testis | – | ND | ND | ND | ND | ND | ND | ND | ND |
| Thyroid | – | ND | ND | ND | ND | ND | ND | – | – |
| Trachea | weak | ND | ND | ND | ND | + | + | + | + |
| Aorta | – | – | – | – | – | – | – | – | – |
| Coronary artery | – | ND | ND | ND | ND | ND | ND | – | – |
| Cornea | ND | ND | ND | ND | ND | + | + | ND | ND |

* 65y, female cadaver.

** Granular staining pattern was noted in glomeruli.

† ND: not done.

‡ (+) = weakly positive.

separation methods were used: (a) pieces of skin were immersed in 1 M NaCl at 4°C for 5 days as described by Scaletta *et al.* (1978), or (b) floated in PBS at 37°C for 5 days as described by Regnier, Prunieras & Woodley (1981). The NaCl was replaced every 24 h. After treatment, the epidermis was separated easily from the dermis.

After separation, samples were embedded in O.C.T. compound and snap frozen, and 6 μm sections were processed for IIF.

### *Fixation of human skin in various solvents*

Cryostat sections (6 μm) of each human skin sample were washed for 15 min in PBS, and then immersed in acetone at −20°C, pure ethanol at room temperature, and 6% formalin at room temperature respectively for 30 min. The sections were then washed for 20 min in PBS and subjected to IIF.

## RESULTS

### *Direct immunofluorescence*

Results of DIF on various organs obtained at autopsy from the patient with EBA are summarized in Table 1. Sharp fluorescent staining for IgG and $C_3$ was found along the BMZ of the skin, tongue, oesophagus and faintly along the trachea. Granular fluorescent staining for IgG and C3 was found in the glomeruli, and this may have been related to the circulating immune complexes previously detected in the patient (Furue *et al.*, 1986).

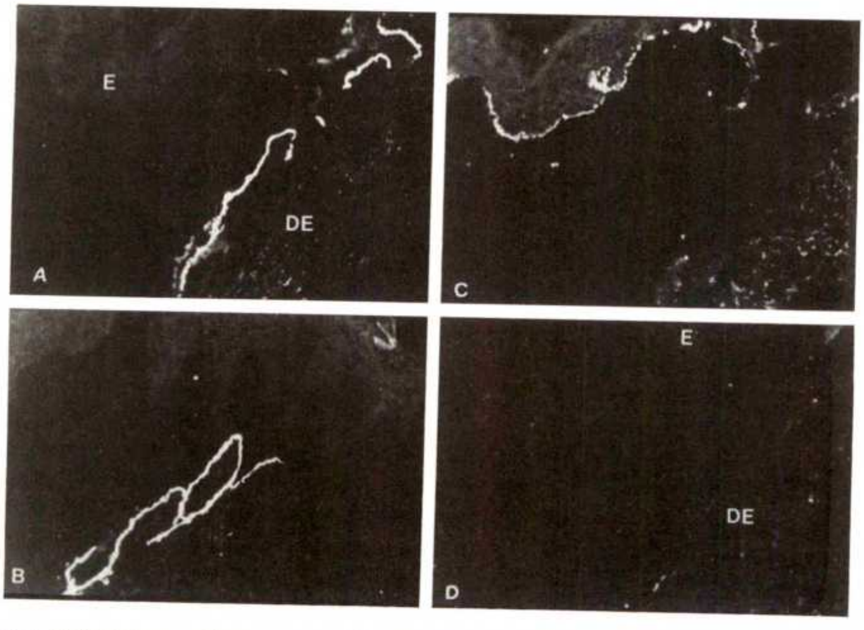

FIGURE 1. Indirect immunofluorescent staining of separated human skin. (A) NaCl-separated skin with EBA serum. Linear fluorescence seen on the dermal side. (B) PBS-separated skin with EBA serum. Linear fluorescence seen on the dermal side. (C) NaCl-separated skin with BP serum. Linear but discontinuous fluorescence seen on the epidermal side. (D) PBS-separated skin with BP serum. Linear fluorescence was not visible on the epidermal or the dermal side. E: epidermis; DE: dermis.

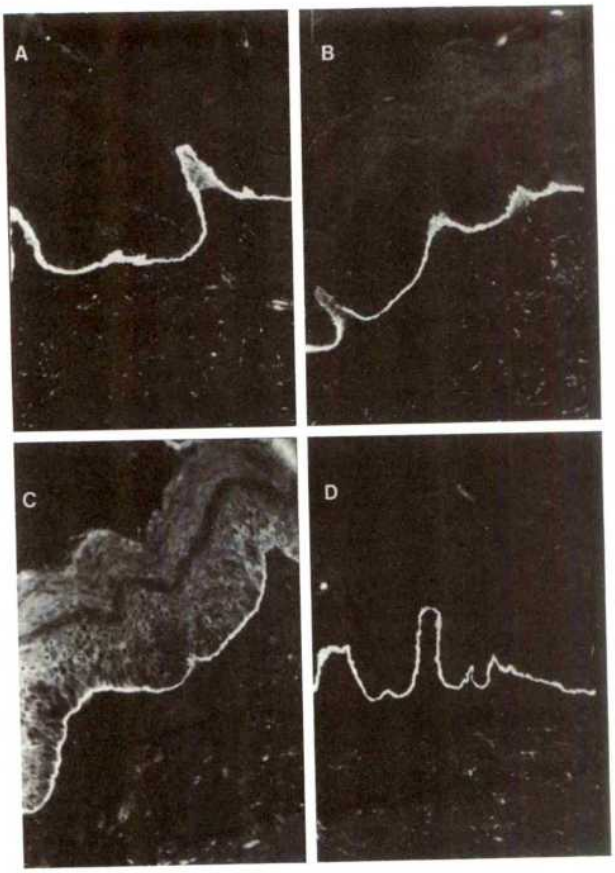

FIGURE 2. IIF staining for EBA antigen in human skin fixed in (A) acetone; (B) ethanol; (C) formalin, and (D) untreated control.

*Indirect immunoelectron microscopy with EBA and BP sera*
The serum from the patient with EBA bound to the upper dermis beneath the basal lamina, while sera from all three patients with BP bound to the lamina lucida along the basal cell plasma membrane.

*Distribution of EBA antigen*
Indirect immunofluorescence staining was performed on tissue samples from various organs of mice, guinea-pigs, rabbits and a human cadaver. Linear BMZ staining was found in the skin, tongue, oesophagus, trachea, cornea and weakly in the bladder (Table 1). All three BP sera showed organ and species specificity very similar to those of EBA serum (Table 1).

*Localization of EBA and BP antigens in separated skin*
EBA serum produced a sharp linear band of fluorescence at the dermal side after separation both in NaCl (Fig. 1A) and in PBS (Fig. 1B). No fluorescence was observed along the epidermal side.

In contrast, BP sera produced a thin, bright linear fluorescent band on the epidermal side after

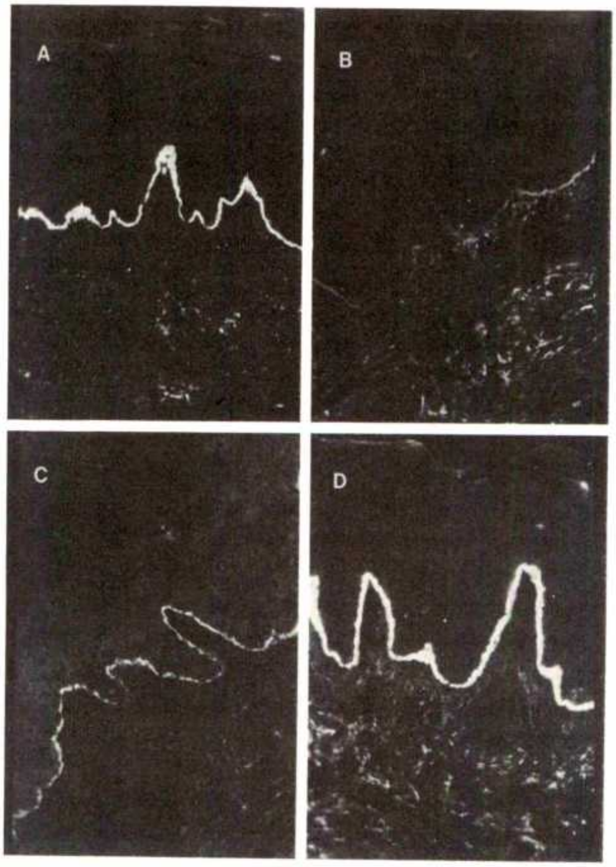

FIGURE 3. IIF staining for BP antigen in human skin fixed in (A) acetone; (B) ethanol; (C) formalin, and (D) untreated control.

separation by cold NaCl. This fluorescence was occasionally interrupted and discontinuous (Fig. 1C). In PBS separated skin, fluorescence with BP sera was not detected (Fig. 1D). However, a very faint fluorescence was detected occasionally on the epidermal side. No fluorescence was observed along the dermal side.

The staining patterns were the same in all of the five skin samples tested with each of the sera. None of the control normal human sera showed positive fluorescence.

### *Antigenic stability of EBA and BP antigens after fixation*

EBA antigen was detected after fixation in acetone (Fig. 2A) and ethanol (Fig. 2B) as strongly as in an untreated control (Fig. 2D). Fixation in formalin reduced the intensity of fluorescence for EBA antigen (Fig. 2C).

BP antigen showed a sharp linear fluorescence after fixation in acetone (Fig. 3A) and in the untreated control (Fig. 3D). However, fixation in ethanol (Fig. 3B) and formalin (Fig. 3C) reduced the intensity of fluorescence. The decrease in the intensity of fluorescence was most marked after fixation in ethanol.

TABLE 2. Dilutions of EBA and BP sera which still detected EBA and BP antigen after fixation of the substrate by acetone ethanol, or formalin

| | Substrate | | | | | | |
|---|---|---|---|---|---|---|---|
| | Human skin | | | | Rabbit cornea | | |
| Fixation | EBA | BP 1 | BP 2 | BP 3* | EBA | BP 1 | BP 2 |
| Acetone | ×320 | ×640 | ×160 | ×640 | ND | ND | ND |
| Ethanol | ×320 | × 10 | × 20 | × 40 | ×160 | ×10 | ×10 |
| Formalin | × 80 | ×160 | × 80 | ×320 | ND | ND | ND |
| Not fixed (control) | ×320 | ×640 | ×160 | ×640 | ×160 | ×80 | ×80 |

ND: not done

* Sera from three patients with BP.

To quantitate the antigenic stability, semi-quantitative studies were performed with each serum by serially titering out the serum from 1:10 to 1:1280 on human skin and rabbit cornea. These results are shown in Table 2. The titres of BP sera (1:640, 1:160, 1:640) for unfixed human skin were reduced markedly after ethanol fixation (1:10, 1:20, 1:40), whereas EBA serum showed the same titre both in unfixed and ethanol-fixed human skin sections (1:320). Apparent loss of the antigenic moiety of BP antigen was also demonstrated when rabbit cornea was used as the substrate (Table 2).

## DISCUSSION

The different binding specificities of the EBA and BP sera found on indirect immunoelectron microscopy are in accordance with previous reports (Holubar *et al.*, 1975; Nieboer *et al.*, 1980). Both EBA antigen and BP antigen were detected in the skin, tongue, oesophagus, trachea, cornea and bladder of the EBA patient, the control human cadaver and several other mammals. The distribution of BP antigen was consistent with that reported previously (Beutner, Jordon & Chorzelski, 1968; Taneda *et al.*, 1979; Danno, Toda & Imamura, 1982).

There have been only a few reports on the anatomical distribution of EBA antigen (Wilson *et al.*, 1980; Ray *et al.*, 1982). Ray *et al.* (1982) found that EBA antigen was present in the oesophagus, but not in the stomach or duodenum when they examined the patient with EBA by endoscopic biopsy. Wilson *et al.* (1980) reported that one of the sera of their patients with EBA contained antibodies to the BMZ of the ducts of the exocrine portion of monkey pancreas, while BP antibodies failed to react. We did not, however, detect EBA antigen in the pancreas of our patient or of a non-affected human cadaver. Our findings indicate that two kinds of BMZ antigens are specifically localized beneath the stratified squamous, pseudo-stratified columnar and transitional epithelia, as opposed to type IV collagen (Yaoita, Foidart & Katz, 1978) and laminin (Foidart *et al.*, 1980) which are present in all BMZ, including vascular tissues.

In another series of experiments, we compared the immunological properties of EBA antigen and BP antigen using variously treated human skin samples.

EBA serum bound exclusively on the dermal side of the skin after DEJ separation by NaCl and PBS, whereas BP sera attached to the epidermal side. The binding ability of BP sera to the skin after PBS treatment was much weaker than that seen after NaCl treatment. This may have been due to elution of BP antigen (Diaz *et al.*, 1977) or protease induction (Woodley *et al.*, 1983) which could influence the BP antigen molecule. Very recently, the differentiation of EBA from BP using NaCl-separated skin was reported by Gammon *et al.* (1984), and our findings are consistent with this. Additionally, we found that the separation of the skin with warm PBS could be used for differentiation of EBA serum from BP serum.

The stability of EBA antigen and BP antigen was examined after fixation in acetone, ethanol and formalin. Acetone did not influence the antigenic stability of these two antigens; however, formalin reduced the stability of both. On ethanol fixation, EBA antigen was stable, whereas the antigenic stability of BP antigen was largely abrogated. These results were confirmed semi-quantitatively in human skin and rabbit cornea. We obtained similar results using rabbit, guinea-pig and mouse skin as substrate (data not shown).

Danno *et al.* (1982) have described the loss of antigenicity of BP antigen after ethanol fixation of human skin. This may be due to elution of BP antigen from the section or denaturation or chemical change resulting in loss of antigenicity. Thus, prefixation of the substrate in ethanol may be a reliable method for differentiating EBA serum from BP serum.

Since immunoelectron microscopy is technically difficult, time-consuming and of limited availability, the easier differential procedures reported in this paper may be useful in classifying the bullous disorders which manifest circulating anti-BMZ antibodies.

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# HB8: a monoclonal antibody recognizing elastic fibre microfibrils

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## SUMMARY

We report an ultrastructural study of the reactivity of an IgG monoclonal antibody, which reacts with human dermal elastic fibres. On electron microscopy, immunogold labelling demonstrated the reactivity of HB8 with the mirofibrillar component of the fibres. In contrast, the electron-dense granular component which contains elastin was unlabelled with HB8. A fine network of microfibrils from the level of the oxytalan fibres to the large elastic fibres of the deep dermis was labelled with gold particles. No reactivity was detected on collagen fibres. These results demonstrate that HB8 reacts with the microfibrillar glycoproteins of the elastic fibres in normal skin. The precise biochemical nature of the antigens identified by HB8 is still unknown.

In skin, the elastic fibres are found in the middle and deep dermis. Ultrastructurally, they consist of electron translucent areas surrounded by microfibrils. However, the relationship between the two elements (the amorphous and the microfibrillar components) is not understood. Microfibrils are invariably present during the process of elastogenesis (Cleary & Gibson, 1983) and they have been claimed to represent a form of pre-elastin (Fahrenbach, Sanberg & Cleary, 1966) and a scaffold on which amorphous elastic tissue is deposited (Ross, 1973; Sanberg, Soskel & Wolt, 1982). However, the two components have different susceptibilities to enzyme digestion (Ross & Bornstein, 1969) and different staining reactions (Greenlee, Ross & Hartman, 1966; Frances & Robert, 1984). The microfibrillar component consists of 10–12 nm microfibrils which are located largely on the surface of mature fibres. Sometimes they are seen lying free in the tissue space or intertwining around and between collagen fibres. They have been described in close relationship with, and as components of, basement membranes (Cleary & Gibson, 1983). In the skin, they are associated with the dermal-epidermal junction and called oxytalan fibres (Fullmer & Lillie, 1958). Definition of the molecular composition of the microfibrils has progressed slowly because of difficulties in solubilization. The amino acid content of the microfibrillar proteins differs entirely from that of

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elastin (Uitto, 1979). On the basis of their affinity for cationic stains, they have been shown to be glycoproteins (Fanning & Cleary, 1985). Recent studies (Sear, Grant & Jackson, 1981; Gibson & Cleary, 1982) have indicated the existence within the microfibrils of a collagen-like component. If these studies are confirmed, the microfibrils must then be considered as a complex of a special type of collagenous glycoprotein with other structural glycoproteins.

From an immunological point of view, the recent development of anti-elastin antibodies has provided a useful tool for the investigation of the biosynthesis, localization and degradation of elastin. Specific double antibody techniques have been developed for identification of elastin both intracellularly and in the extracellular matrix at the light and ultrastructural levels (Damiano *et al.*, 1979, 1984).

Immunohistochemical studies of normal skin using anti-amyloid-P component serum have suggested that some of the structural glycoproteins of the microfibrils have antigenic properties similar to those of the amyloid-P component (Breathnach *et al.*, 1981a, b, 1982, 1983).

We have recently produced a new monoclonal antibody, HB8, by immunizing BALB/c mice with human lymphocytes. This has been observed to react with elastic fibres, possibly with the microfibrillar component (Brochier *et al.*, 1984; Dawson *et al.*, 1984). We report here a study of HB8 reactivity using an indirect immunogold technique. This method has enabled us to demonstrate the reactivity of HB8 with the microfibrillar component of the dermal fibres, and the oxytalan fibres of the dermal-epidermal junction.

## METHODS

### *Preparation and characterization of HB8*

The monoclonal antibody HB8 was synthesized using the technique of cellular hybridization, by immunizing Balb/c mice with a suspension of lymphoid cells obtained from the human thoracic duct. A variety of monoclonal antibodies was obtained and these were tested to determine their cross-reactivity with human and animal tissue (Brochier & Schmitt, 1985). One cell line secreted the IgG HB8, which was found to label elastic tissue (Brochier *et al.*, 1984).

### *Immunoelectron microscopy*

Labelling experiments were carried out on samples of normal human skin obtained from plastic surgery (abdominal, axillary and breast skin) and snap frozen in liquid nitrogen. Frozen sections of 15 m$\mu$ were cut, air dried and fixed in cold acetone (−20°C) for 10 min.

Indirect immunogold staining was performed as follows: sections were incubated with HB8 (diluted 1:50) for 45 min at 37°C, or with control antibodies or normal mouse serum at equivalent protein concentrations, and washed twice in phosphate-buffered saline (PBS) pH 7·2, for 15 min. The sections were then incubated with gold particle-conjugated second layer antiserum (goat anti-mouse Ig labelled with gold granules of 20 nm diameter; dilution 1:2, Janssen, Beerse, Belgium) for 45 min at 37°C, and again washed twice in PBS.

The sections were prepared for electron microscopy by fixation in 2% glutaraldehyde, post fixation in 1% osmium tetroxide, dehydration, and embedding in epoxy medium. Ultrathin sections were stained with lead citrate and uranyl acetate and examined with a Philips EM300 electron microscope.

*Controls*. Normal non-immune mouse serum and anti-keratin monoclonal antibodies (KL1) were used in parallel with HB8 in order to determine the specificity of the reactions.

## RESULTS

On electron microscopy the most frequent observation was the localization of gold granules attached to the microfibrillar component of the dermal elastic fibres. The particles appeared as isolated granules or small clusters irregularly located on the microfibrils (Fig. 1). Occasional gold particles were seen between collagen fibres. No HB8 reactivity was detected in the central amorphous part of the fibres.

From the deep dermis to the dermal-epidermal junction, elongated thin microfibrils travelling between collagen bundles were densely labelled with gold particles (Fig. 2). Some of them were continuous with large dermal elastic fibres (Fig. 1, arrow).

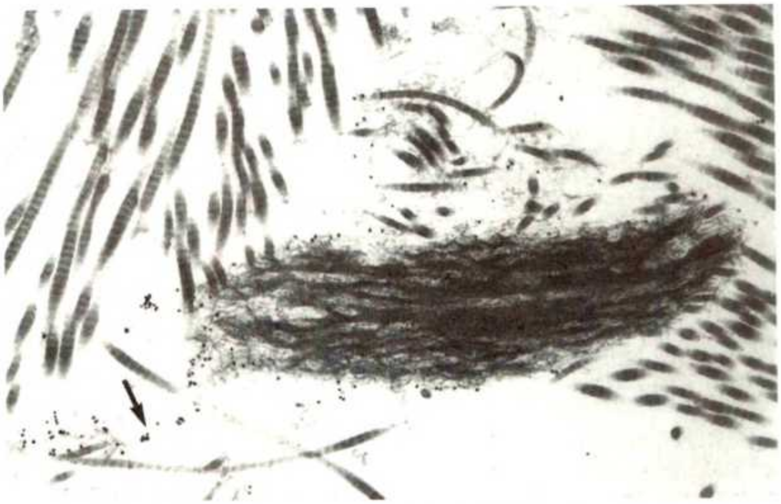

FIGURE 1. Section of normal human skin debris, stained with HB8. Note the presence of gold particles on the elastic fibre microfibrils, and the absence of non-specific labelling of collagen fibres (arrow = small fibres in close association with the elastic fibre) (original × 32 000).

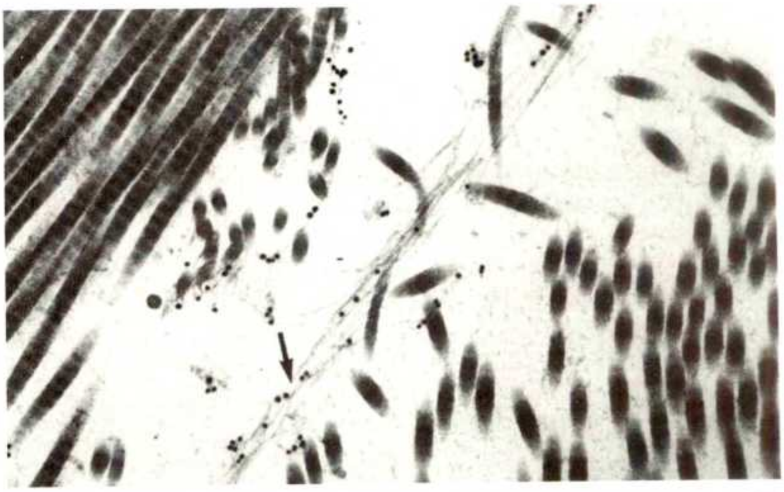

FIGURE 2. In the dermis a fine network of microfibrils (arrow) was specifically labelled by HB8 and gold granules (original × 51 000).

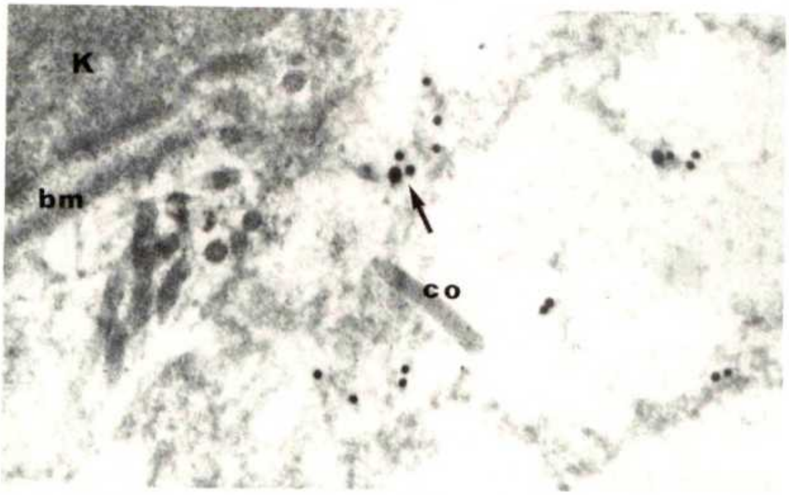

FIGURE 3. At the dermal-epidermal junction, HB8 labelled fine microfibrils which correspond to oxytalan fibres (arrow) forming a complex interlacing network with isolated collagen fibres. K = basal keratinocytes; bm = basement membrane, co = collagen fibres (original × 88 000).

At the dermal-epidermal junction, HB8 antibody labelled small microfibrils in close proximity to the basement membrane (Fig. 3). These microfibrils, corresponding to the oxytalan fibres, formed a complex interlacing network with isolated collagen fibres. The density of gold granules fixed on these oxytalan fibres was lower than that observed on microfibrils in the middle dermis and on elastic fibres. No reactivity was observed with the other components of the basement membrane zone.

At the ultrastructural level, no labelling was obtained with the control sera.

## DISCUSSION

The most striking observation in our ultrastructural analysis was the reactivity of HB8 with the microfibrillar component of the dermal elastic fibres. Our data are similar to those recently reported by Kambe *et al.* (1985). This group produced a monoclonal antibody (NKH-1) by immunization of mice with junctional interface components extracted from skin by urea and mercapto-ethanol. This antibody labelled dermal elastic fibres and the dermal-epidermal junction, and an immunoelectron microscopic study using the immunoperoxidase technique revealed that the antigen reacting with NHK-1 was localized on the microfibrils of papillary oxytalan fibres and the microfibrillar mantle surrounding the mature elastic fibres.

HB8 and NHK-1 are characterized by their absence of reactivity with the central amorphous part of elastic fibres which contains elastin. The absence of a relationship between the two antigens used to immunize the mice (lymphocytes and skin extract) favours the hypothesis that NB8 and NHK-1 recognize small epitopes expressed on glycoproteins associated with elastic fibre microfibrils (Fanning & Cleary, 1985). However, it is necessary to confirm that the absence of reactivity with the amorphous matrix of the elastic fibres is not due to a lack of reagent penetration into this dense structure.

We have reported in a previous study (Dawson *et al.*, 1984) the good correlation between the elastic fibre labelling with HB8 and the staining with orcein using sections of normal human skin. We have recently obtained good results with HB8 in quantitative analysis of skin ageing processes (N.Ibtissam, unpublished data). HB8 may have practical applications for the anatomist, histopathologist and comparative biologist.

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# Contact and photocontact allergy to musk ambrette

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## SUMMARY

Musk ambrette, a contact and photocontact allergen, is a synthetic fragrance present in aftershave lotions and many toiletries. Thirty-four patients with contact and photocontact allergy to musk ambrette were studied. All had a facial eczema. Four distinctive clinical pictures were seen. These were plaques of eczema, jawline (mandibular) dermatitis, acute contact dermatitis and chronic actinic dermatitis. Twenty-six patients were light sensitive by history and 10 were diagnosed by clinical criteria as having chronic actinic dermatitis. Patch and photopatch testing to musk ambrette showed that seven patients had pure photocontact dermatitis to musk ambrette. Eight patients had contact dermatitis to musk ambrette but this was exacerbated by irradiation. Cross reaction with moskene occurred. Scrupulous avoidance of musk ambrette has resulted in clinical resolution in most patients.

Musks are important fragrance compounds in perfumes. Natural musks (muscone, civettone) are now too expensive to be used commercially, so synthetic musks are used. Musk ambrette is the chief synthetic musk in perfumed products (Fig. 1), the annual production being estimated at approximately 700 tonnes. Other musks are musk ketone (250 tonnes), musk xylene (450 tonnes), musk tibetene (5 tonnes) and moskene (75 tonnes) (Fig. 1). Musk ambrette has a dual function, as a fragrance and as a fixative to prolong the duration of the fragrance. It is used in aftershave preparations, colognes and women's perfumes. It is present in many toiletries, including shaving creams and soap, hair creams and sprays and lotions, and also in perfumed topical medicaments. It is a permitted food additive and is mainly used to simulate blackberry flavour and in certain highly perfumed sweets.

Epstein (1969) described photocontact allergy to male perfumes but was unable to identify the photoallergen. The first report of photocontact allergy to musk ambrette was in 1979 (Raugi, Storrs & Larsen, 1979) and there have been isolated case reports since then (Kroon, 1979; Giovinazzo *et al.*, 1980; Burry, 1981; Zugerman, 1981; Ramsay, 1984) and previous brief reports of some of the patients described in this paper (Wojnarowska, Calnan & Hawk, 1982; Cronin, 1984).

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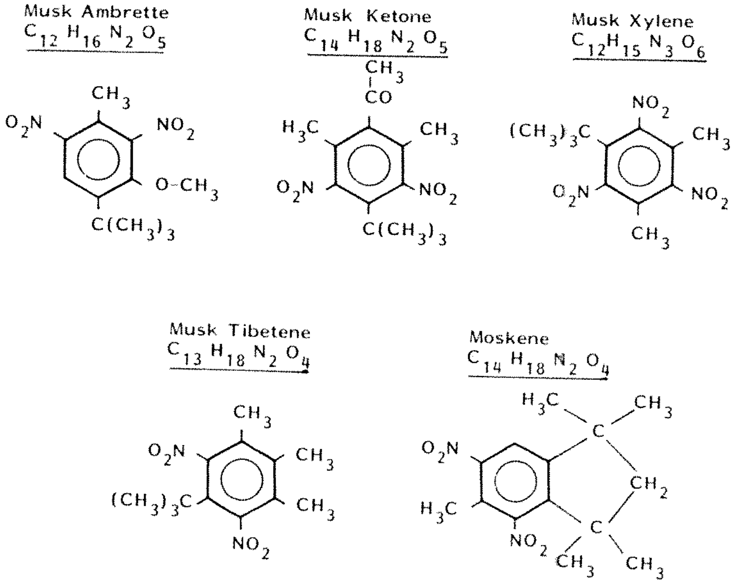

FIGURE 1. Structure of musk ambrette, musk ketone, musk xylene, musk tibetene and moskene.

## METHODS

### *Patients*

Photopatch testing with musk ambrette was commenced at St John's Hospital in 1979. It was performed routinely on all men with a facial dermatosis and all patients with a diagnosis of chronic actinic dermatitis.

Thirty-four patients had positive patch and photopatch tests to musk ambrette during the period 1979–1982. History, clinical features and photosensitivity were reviewed, and a detailed questionnaire administered to determine their past and present usage of compounds possibly containing musk ambrette. The musk ambrette content of these compounds was then confirmed with the manufacturers.

### *Patch and photopatch testing*

All the patients were tested with the European standard series of allergens (Trolab, Copenhagen, Denmark). All patients with a clinical diagnosis of chronic actinic dermatitis, referred from the Department of Photobiology, were also tested for sensitivity to the standard photopatch test series, which included chrysanthemum but not other compositae (Barber & Cronin, 1984). Musk ambrette 5% in petrolatum is a component of the standard St John's Hospital photopatch series. It was applied as a standard patch test, and as a photopatch test with irradiation by long wave ultraviolet light (UVA) at 48 h. The source of UVA was either a Phillips UVA bank TL20W/08 (25 min at 30 cm), or a Kromayer lamp with Wood's glass filter as a contact source for 5 min. The output of both sources was measured with an International Light TL442A phototherapy radiometer calibrated by the National Physical Laboratory. The total dose of ultraviolet light was approximately 0·5 J/cm² for both sources. Readings were made at 48 h and 96 h.

Sixteen patients were studied in more detail. The standard patch test, photopatch test with UVA irradiation at 48 h, and a patch from which all light was excluded for 48–96 h by use of black paper and black polythene were compared to determine whether musk ambrette was a contact allergen or a photocontact allergen. A further patch test was exposed to UVA 5 min after application to determine whether a photo-decomposition product was the allergen. Twenty patients were studied with patch and photopatch tests using musk xylene, musk ketone and moskene (each at 5% in petrolatum) to establish whether cross sensitization to other musks was present.

## RESULTS

Thirty-four patients had positive patch tests to musk ambrette during the period of study. This was less than 5% of all male patients with a facial dermatosis, 10% of those who were photopatch tested and 9% of all patients with a diagnosis of chronic actinic dermatitis. They were all men. There were seven (20%) Asian patients. The Asian patients were relatively young (30–46 years, mean 40 years), whereas the European patients were much older (39–79 years, mean 61 years). There were occupational differences in the groups. There were more professional men among the Asians; this may reflect differences in social usage of perfumes.

The duration of the eruption in individual patients varied from a few months to 10 years, with an average of 4 years.

### *Clinical features*

The eruption was eczematous. The initial stages were erythematous, and when acute often oedematous, weeping and crusted. Two patients were diagnosed as impetigo at this stage. The chronic eruption was scaly and erythematous, the skin thickened and sometimes lichenified. In Asian patients, post-inflammatory hyperpigmentation was prominent and persistent, even mimicking a fixed drug eruption.

There were four main patterns of distribution: plaques, jaw-line (mandibular) dermatitis, acute contact dermatitis, and chronic actinic dermatitis. The light exposed areas, e.g. face, neck and backs of hands were chiefly affected. The pattern was not fixed and continued exposure or exposure to aerosols resulted in a change from a localized to a generalized facial dermatitis.

In the first pattern, plaques of eczema were scattered over the cheeks, sides of the neck and occasionally the forehead (Fig. 2). This was the most common pattern in the Asian men, occurring in five of the seven. This was initially diagnosed as fixed drug eruption, discoid eczema or lupus erythematosus. This pattern was less common in the European men, but did occur.

In the jaw-line pattern, a zone of eczema was present corresponding to the area of application of an after-shave, extending along the jaw-line and across the lower part of the ear, with an area of the neck both behind and below the ear (Fig. 3). Initial diagnoses included seborrhoeic dermatitis, psoriasis and, in one very acute case, impetigo. This was the commonest pattern in the European men (15 of 27). It was reported in retrospect by some patients who at the time studied had the picture of chronic actinic dermatitis.

Five patients experienced episodes of acute contact dermatitis of the face, with involvement of the whole face and marked swelling of the eyelids (Fig. 4). In one patient it was mistaken for impetigo. These episodes were caused in at least two patients by exposure to aerosols of perfume or aftershave lotion.

The fourth pattern was a picture clinically indistinguishable from chronic actinic dermatitis present in 10 patients. The whole face apart from the periorbital area was involved with a chronic light exacerbated dermatitis with lichenification in some cases. There was often

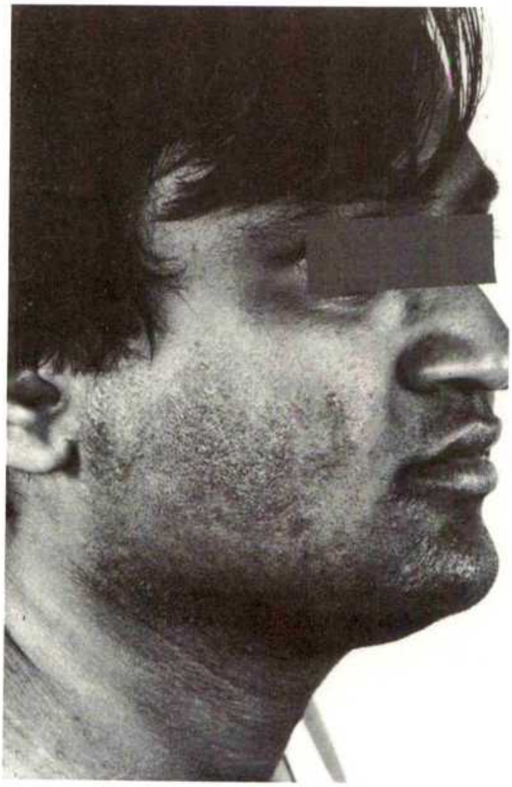

FIGURE 2. Plaque pattern of eruption.

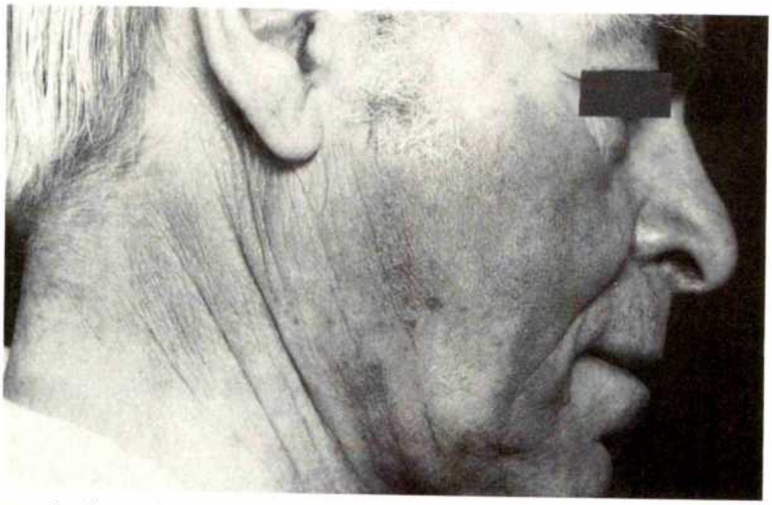

FIGURE 3. Jawline pattern of eruption corresponding to the area of application of aftershave.

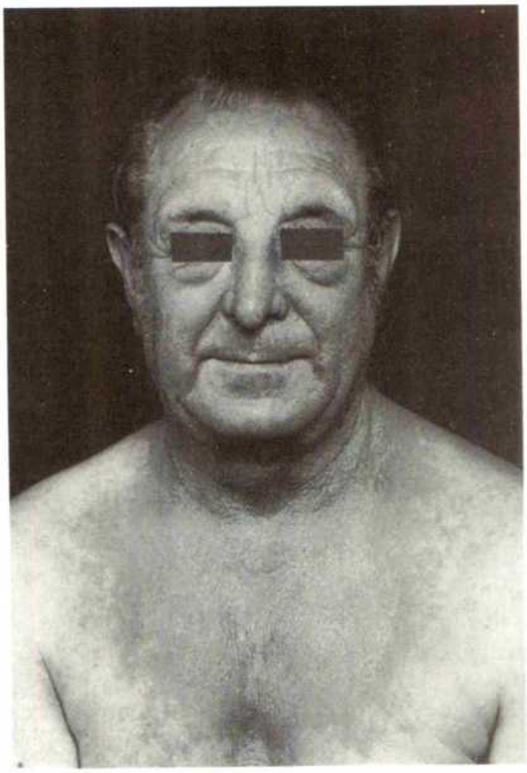

FIGURE 4. Acute contact dermatitis after exposure to an aerosol.

extension behind the ears and below the chin. Some of these patients initially had a more localized form. These patients all had abnormal monochromator tests.

These patterns varied from time to time within patients depending on degree of exposure, treatment and chronicity. Continued exposure often culminated in the picture of chronic actinic dermatitis.

Sites other than the face were involved; most commonly the hands (74%), particularly the backs of the hands and finger webs, probably because of aftershave trickling from the palms between the fingers during application. The other areas affected were the arms (42%), the trunk (31%), the legs (26%), and the axillae (20%), due to deodorant usage.

### *Illustrative case history*

A 46-year-old Indian dentist had had psoriasis for many years. For 3 years he had scaling and itching in the beard area which was attributed to psoriasis; he regularly used an aftershave lotion. In January he visited Los Angeles and purchased a new aftershave lotion there. The weather was sunny and he developed an acute weeping dermatitis of the face, with a patch of eczema on the forearm. He was patch tested and found to be positive to musk ambrette, hydroxy citronellol and oak moss. His aftershaves and soap contained musk ambrette. The patch of eczema on his forearm corresponded to an area of contact with his patients' beard area and presumably their aftershaves, while he was attending to them in the surgery. Three months later in April he developed a recurrence after gardening on a bright day. He has had two further recurrences. These episodes may have been related to the use by his wife of perfumes and soap which are known to contain musk ambrette. With scrupulous avoidance of all perfumes the eruption in the beard area has cleared and the acute dermatitis has not recurred, but he has been left with disfiguring post-inflammatory hyperpigmentation.

### *Patch testing to musk ambrette*

All 34 patients had a positive patch test to musk ambrette at 96 h. Positive reactions to musk ambrette are rare and it did not elicit a positive reaction in the majority of patients with a facial dermatosis, or in 90% of patients with chronic actinic dermatitis.

Sixteen patients were studied in more detail using irradiated and covered patch tests to determine whether musk ambrette was a contact or a photocontact allergen. Eight patients manifested pure photocontact allergic dermatitis. Seven of these reacted to musk ambrette only after exposure to UVA irradiation 48 h after application of the patch. Eight patients reacted to the application of musk ambrette from which all light had been excluded for 96 h, and thus represented ordinary allergic contact dermatitis. However, in six of the patients the reaction was much stronger when the patch test was exposed to UVA irradiation at 48 h than when it was covered.

The dose of UVA used for photopatch testing elicited a photo-reaction (erythema) in only two patients, and the response to musk ambrette, which was papular, was clearly distinguishable from the surrounding erythematous reaction.

The result of patch and photopatch testing to other musks is shown in Table 1. Cross reactions did occur and were most common with moskene (38%). The synthetic process employed makes contamination of moskene with musk ambrette most unlikely.

The most common allergens other than musk to which positive reactions were observed in the group of patients were perfume mix (34%) and balsam of Peru (20%) (Table 2); 54% reacted to one or both of these mixtures. The other allergens were positive in less than 10%, except Uvistat which gave an irritant reaction in 20% of patients (Table 2). One patient had a positive patch test to chrysanthemum.

Twenty-six patients described light sensitivity. They had noticed provocation or exacerbation of the rash by sunlight, in one patient by fluorescent lights, and in another by arc welding. In some patients who drove a car frequently the right side of the face or right hand were more severely affected. Sixteen patients had a patchy eruption that worsened with exposure to UV radiation. Ten patients had the clinical picture of chronic actinic dermatitis with involvement of all the light exposed areas except the periorbital area, and abnormal light tests. Twenty-one of the light-sensitive patients had monochromator tests, 10 were abnormal. The results will be described in a separate paper. Eight patients had not noted any relationship to sunlight.

TABLE 1. Patch and photopatch test reactions to musks

| Type of musk (no. of patients tested) | Covered patch test no. positive | Photopatch test no. positive |
|---|---|---|
| Musk ambrette ($n = 16$) | 8 | 16 |
| Musk xylene ($n = 21$) | 0 | 1 |
| Musk ketone ($n = 21$) | 0 | 1 |
| Moskene ($n = 21$) | 1 | 8 |

TABLE 2. Results of patch and photopatch tests

| | No. of patients | |
|---|---|---|
| | Tested | Positive |
| *Patch tests* | | |
| Perfume mix | 34 | 12 |
| Cinnamyl alcohol | 16 | 2 |
| Cinnamaldehyde | 16 | 5 |
| Oak moss | 16 | 4 |
| Hydroxycitronellol | 16 | 2 |
| Isoeugenol | 16 | 3 |
| Atranorin | 16 | 2 |
| Balsam of Peru | 34 | 7 |
| Nickel | 34 | 2 |
| Colophony | 34 | 1 |
| PPD | 34 | 2 |
| MBT | 34 | 1 |
| Potassium dichromate | 34 | 2 |
| Wool alcohols | 34 | 1 |
| Epoxy resin | 34 | 2 |
| Neomycin | 34 | 2 |
| Dowicil 200 | 34 | 1 |
| Parabens | 34 | 1 |
| Thiuram mix | 34 | 1 |
| Chrysanthemum* | 17 | 1 |
| Uvistat* | 17 | 7 |
| Escalol* | 17 | 1 |
| Phosphorous sesquisulphide* | 17 | 1 |
| *Photopatch tests* | | |
| Musk ambrette | 34 | 34 |
| Promethazine* | 17 | 4 |
| Chlorpromazine* | 17 | 4 |
| Trichlorcarbanilide* | 17 | 1 |
| Sulpha-mix* | 17 | 1 |

* Done in all patients with suspected chronic actinic dermatosis and clinical photosensitivity.

A detailed questionnaire was returned by 32 of 34 patients. This included details of past and present usage of male toiletries, and usage of perfumes by their wives or girlfriends. The presence of musk ambrette in these products was then ascertained from the manufacturers. Almost all the aftershaves contained musk ambrette. It was also present in many women's perfumes, in shaving products, talcum powders, deodorants, hair creams, hair sprays, shampoos, soaps and baby products.

All patients had initially used one or more aftershave lotions containing musk ambrette. The biggest selling brand in the U.K. was used by 21 patients, and one patient had used eight brands. In many men the problem persisted despite abandoning the use of aftershave because they unknowingly continued to use products containing musk ambrette. Although the most common were shaving products, hair creams or sprays and baby products, one patient worked in a pharmacy where perfume sprays were sampled by customers. In some patients, exposure to wives' or girlfriends' perfumes was a cause of both acute exacerbations and chronic dermatitis.

In many patients the scrupulous avoidance of all sources of musk ambrette has returned their skin to normal.

Seventeen patients replied to a postal follow-up questionnaire. Thirteen reported their skin to be normal. Four were improved compared with the previous year. In addition to these 17, one patient had died of carcinoma and one was too ill to fill in the questionnaire.

## DISCUSSION

This study demonstrates that contact and photocontact allergy to musk ambrette are both more common than previously reported, (Epstein, 1969; Raugi *et al.*, 1979; Kroon, 1979; Giovinazzo *et al.*, 1980; Burry, 1981; Zugerman, 1981; Addo *et al.*, 1982; Ramsay, 1984). Although these thirty-four patients comprise less than 1% of all patients patch tested in our clinic, they are approximately 10% of photopatch test patients and 9% of those diagnosed clinically as having chronic actinic dermatitis (Barber & Cronin, 1984).

We have confirmed previous reports (Kroon, 1979; Burry, 1981; Addo *et al.*, 1982) that musk ambrette can act as a contact allergen, and Burry's report that this allergy is exacerbated by UV irradiation (Burry, 1981). In those patients in whom musk ambrette was a pure photocontact allergen the majority showed marked photosensitivity. Photocontact allergy to moskene has been found both in this study and previously by Raugi *et al.* (1979) and Cronin (1984), and to musk xylene by Cronin (1984). Photocontact allergy to musk ketone has not previously been demonstrated.

There have been reports of induction of the state of persistent light reaction by musk ambrette (Giovinazzo, 1980; Zugerman, 1981; Ramsay, 1984) and in our own St John's patients (Wojnarowska *et al.*, 1982; Cronin, 1984). Seven of our patients had the clinical picture and abnormal responses to light consistent with this, but we were unable to satisfy ourselves that any of them had not been subjected to continued inadvertent exposure because of the wide range of products containing musk ambrette and a long-term study is currently in progress to determine the outcome in these patients. The same may be true of the previously reported patients. As in all the previous reports, the patients were male. There are several reasons why men are affected almost exclusively. The concentration of musk ambrette used in aftershaves can be as high as 15% (Giovinazzo *et al.*, 1980). This is applied in large volumes to the thin skin of the face, freshly abraded by shaving and on a maximally light exposed area. This is similar to the method used for photosensitization of guinea-pigs (Kochevar *et al.*, 1979; Giovinazzo *et al.*, 1981). Women's perfumes contain lower concentrations and are applied usually in small volumes to the neck, chest and arms, rather than the face.

Musk ambrette has been in use in toiletries for 60 years but it is only in the last 15–20 years that contact dermatitis has been reported. During this period aftershaves have been marketed, heavily promoted and widely used. Sensitization seems to occur chiefly with the use of aftershaves, but the dermatitis is perpetuated by the use of toiletries containing lower concentrations of musk ambrette once sensitization has occurred. Presumably the use of these lower concentrations on intact skin did not induce sensitization. The use of musk in household products does not seem to initiate contact allergy but could perpetuate it.

The possible sources of exposure to musk ambrette are numerous. It is not practical to compile a list of musk ambrette-free products, because components of products change. Patients who are sensitive to musk ambrette should be warned to use only products specifically stated to be non-perfumed and should also be warned of the dangers of aerosols of other peoples' perfumes or aftershaves, since these resulted in acute episodes in four patients and in one of the original patients of Raugi *et al.* (1979). It is not yet clear whether ingestion of musk ambrette in foods is a serious problem for those patients who have been topically sensitized.

It is hoped that by early recognition of musk ambrette sensitivity and avoidance of the allergen, the number of patients progressing to persistent photosensitivity and chronic actinic dermatitis will decrease. There have been fewer new patients with musk ambrette photocontact allergy presenting to our clinic in the year 1983–1984. We presume that this reflects the recommended reduction in the concentration of musk ambrette, which is now limited to approximately 0·005% in the final product, its complete removal from aftershaves.

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# Immunohistological reactivity pattern of the anti-cutaneous T-cell lymphoma antibody BE2

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## SUMMARY

It has been reported that the monoclonal antibody BE2, raised against leukaemic T cells, reacts specifically with malignant lymphoid cells and represents a valuable reagent for the early identification of cutaneous T-cell lymphomas. To test this hypothesis, a comprehensive range of nodal and cutaneous biopsies have been examined immunohistologically using single and double immunoenzymatic and immunofluorescent staining methods. BE2 showed a broad range of reactivity, consistently labelling normal endothelial cells, B-lymphocytes in mantle and marginal zones, T-lymphocytes in the paracortex of lymph nodes and medulla of thymus, as well as a variety of different macrophage types, including Langerhans cells and dermal macrophages. Furthermore, although BE2-positive T-lymphocytes were most frequent in malignant lymphomas, they were also found in four of 19 benign dermatoses. We conclude that BE2 is neither T-cell nor tumour-cell specific, and that use of this reagent on tissue sections is unlikely to improve the diagnosis of cutaneous lymphomas.

The recognition of the early stages of the cutaneous T-cell lymphomas is difficult for both clinicians and histopathologists. Although the recent introduction of monoclonal antibodies into routine pathology departments has provided considerable information about the phenotype of cutaneous lymphomas, the hope that they would aid in the early diagnosis of these disorders has not been fulfilled. This is because early neoplastic infiltrates are virtually identical with benign dermatoses in terms of their reactivity with currently available antibodies against normal lymphoid cells and their subsets (Chu *et al.*, 1984; Haynes, Hensley & Jegasothy, 1982; Ralfkiaer *et al.*, 1985; Willemze *et al.*, 1983).

Recently, it has been claimed that the monoclonal antibody, BE2, raised against malignant T cells, reacts specifically with malignant lymphoid cells and should therefore aid the early identification of cutaneous T-cell lymphomas (Berger *et al.*, 1982). To test this hypothesis, we have examined normal, benign and malignant cutaneous and nodal biopsies for reactivity with BE2, using immunohistological labelling techniques.

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## METHODS

### *Tissues*

Skin punch biopsies were obtained from patients attending the Dermatology Department of the Finsen Institute. The diagnoses are given in Table 1. Contact allergic reactions were implicated in the pathogenesis of all eczema and patch test cases. In parapsoriasis, the terminology proposed by Lampert (1981) was followed. Normal skin biopsies were obtained from healthy adult volunteers. Samples of normal, hyperplastic and malignant lymphoid organs (tonsil, lymph node, spleen and thymus) were drawn from the frozen tissue bank of the Department of Pathology, the Finsen Institute. In the malignant lymphomas, diagnosis was made in accordance with histological (Lennert *et al.*, 1978) and immunophenotypic criteria (Stein *et al.*, 1984) using standard panels of monoclonal antibodies against lymphoid cells and their subsets as described previously (Ralfkiaer *et al.*, 1985).

### *Processing of fresh tissue specimens*

Biopsies were frozen and stored in liquid nitrogen. 6 μm cryostat sections were lyophilized at −20°C for 2 h, fixed in acetone for 10 min and either stained immediately or wrapped in aluminium foil and stored at −20°C until staining (Stein *et al.*, 1982). In the latter instance, sections were allowed to warm to room temperature prior to unwrapping.

### *Antibodies and immunohistochemical reagents*

Details of the monoclonal antibodies used in this study are given in Table 2. For each antibody,

TABLE 1. Reactivity of BE2 with cutaneous biopsies

| Diagnosis | No. of samples | BE2-positive | BE2-negative* |
|---|---|---|---|
| Normal skin | 9 | 0* | 9* |
| Benign dermatoses | | | |
| Patch tests infiltrates | 4 | 1 | 3 |
| Chronic eczema | 3 | 1 | 2 |
| Psoriasis | 3 | 0 | 3 |
| Lichen planus | 5 | 2 | 3 |
| Cutaneous lymphocytoma | 4 | 0 | 4 |
| Pre- or pseudo-malignant disorders | | | |
| Lymphomatoid papulosis | 5 | 0 | 5 |
| Actinic reticuloid | 5 | 2 | 3 |
| Large plaque parapsoriasis | 8 | 4 | 4 |
| Malignant lymphomas | | | |
| Mycosis fungoides | 18 | 12 | 6 |
| Sézary syndrome | 6 | 3 | 3 |
| Large cell lymphoma (T-cell type) | 4 | 2 | 2 |
| Large cell lymphoma (B-cell type) | 2 | 2 | 0 |
| True histiocytic lymphoma | 1 | 1 | 0 |
| Histiocytosis X | 1 | 1 | 0 |

* Refers to staining of benign or malignant lymphocytes. Labelling of Langerhans cells, dermal macrophages and endothelial cells was observed in all specimens.

TABLE 2. Details of monoclonal antibodies used in the study

| Antibody | Immunoglobulin subclass | Specificity | Source or reference |
|---|---|---|---|
| BE2 | IgG1 | 78 kd tumour cell associated antigen | Berger *et al.*, 1982 |
| Anti-Leu1 | IgG2a | CD5 pan-T-cell antigen | Becton Dickinson |
| OKT6 | IgG1 | CD1 antigen on immature T cells and Langerhans cells | Ortho Diagnostics |
| KB90 | IgG1 | p150,95 molecule on macrophages | D.Y. Mason, Oxford University |

the optimal dilution was established by preliminary titration. Antibodies OKT6, KB90 and anti-Leu1 were all used at a dilution of 1:20. ; for BE2, the optimal dilution was 1:900 for the immunoenzymatic staining procedures, but 1:300 for the immunofluorescence labelling techniques. Immune complexes of alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase (APAAP complexes) were prepared as described previously (Cordell *et al.*, 1984). Unconjugated rabbit anti-mouse immunoglobulin, peroxidase-conjugated rabbit anti-mouse immunoglobulin, peroxidase-conjugated swine anti-rabbit immunoglobulin and fluorescein conjugated F(ab′)$_2$ fragments of rabbit anti-mouse immunoglobulin were all purchased from Dakopatts, Copenhagen, Denmark. Fluorescein-conjugated rabbit anti-mouse IgG2a and rhodamine-conjugated rabbit anti-mouse IgG1 were kindly supplied by Serotec, Birmingham, U.K. Levamisole, naphthol AS-MX, New Fuchsin and 3-ethyl-9-aminocarbazole were all obtained from Sigma Chemical Company.

### *Immunoenzymatic staining methods*

Sections were incubated with monoclonal antibody and stained using a three-stage immunoperoxidase method (Stein *et al.*, 1982) or the alkaline phosphatase:anti-alkaline phosphatase (APAAP) technique (Cordell *et al.*, 1984). In the latter instance, incubation with rabbit anti-mouse immunoglobulin and APAAP complexes was performed twice (Cordell *et al.*, 1984) to increase the sensitivity of the staining procedure. The peroxidase label was revealed using $H_2O_2$ and 3-ethyl-9-aminocarbazole, and the alkaline phosphatase label using a naphthol AS-MX/New Fuchsin substrate (Cordell *et al.*, 1984). Endogenous tissue alkaline phosphatase activity was inhibited by adding 1 mM levamisole to the substrate solution (Ponder & Wilkinson, 1981). Co-expression of BE2, the CD1 antigen (antibody OKT6) and the p150,95 molecule (antibody KB90) was assessed by examination of serial sections.

### *Single and double immunofluorescence staining techniques*

Single staining was performed by a two-stage immunofluorescence method using F(ab′)$_2$ fragments of fluorescein-conjugated rabbit anti-mouse immunoglobulin as the second layer. For the double staining, sections were incubated with a mixture containing equal amounts of BE2, which is an IgG1 antibody, and anti-Leu1, an IgG2a antibody (Table 2), washed and then stained using a mixture of the fluorescein- and rhodamine-conjugated rabbit antibodies against mouse IgG2a or IgG1 immunoglobulin subclasses. These sections were mounted in glycerol phosphate buffer and analysed on the same day in an epi-illumination fluorescence microscope.

### *Control preparations*

Negative controls were performed by omitting the primary monoclonal antibodies and by substituting an IgG2a reagent for the IgG1 antibody and vice versa. The specificity of labelling in the double stained sections was also assessed by comparison with cellular reactivities in single stained serial sections.

## RESULTS

### *BE2-positive cutaneous cell types*

Examination of single stained sections using both the APAAP and the immunoperoxidase methods revealed that BE2 reacted consistently with several morphologically distinct cutaneous cell types, including endothelial cells and cells of dendritic morphology present in epidermis and dermis (Fig. 1). Additionally, in some biopsies BE2 also labelled cells with more regular contours (Fig. 1). These latter cells, which resembled lymphocytes, showed a surface membrane reactivity pattern, whereas the dendritic cells, which resembled macrophages or Langerhans cells, showed strong cytoplasmic positivity (Fig. 1). Identical staining results were obtained with a two-stage immunofluorescence staining method using $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulin as the second layer. However, this technique required a lower BE2 dilution than optimal for immunoenzymatic staining (1:300 as compared to 1:900; see Methods).

While the BE2-positive endothelial cells could be recognized confidently due to their distinctive morphology, confirmation of the lineages of the remaining BE2-positive cutaneous cell types required assessment of serial or double stained sections or both. These experiments

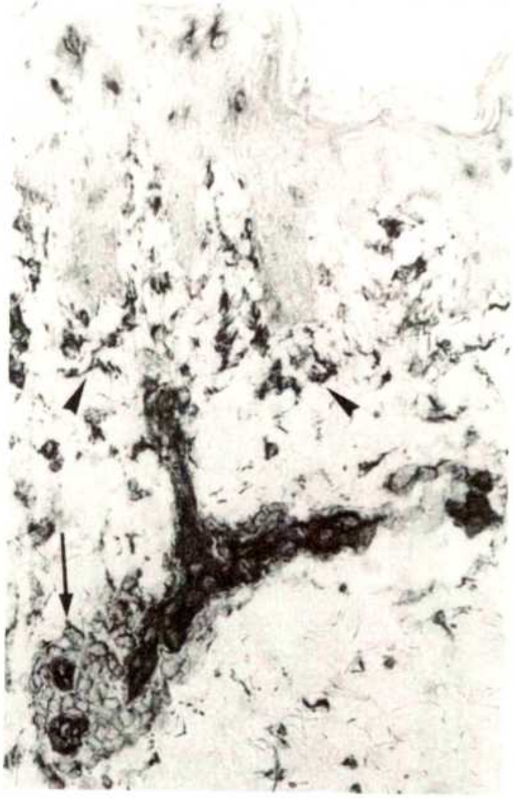

FIGURE 1. BE2-positive T cells (arrow), Langerhans cells, dermal macrophages (arrowheads) and endothelial cells, in a patch test infiltrate. (Original × 400).

demonstrated that BE2 did indeed label Langerhans cells (positive for the CD1 antigen recognized by antibody OKT6) and dermal macrophages (positive for the p150,90 molecule recognized by antibody KB90), and in some instances also cutaneous T-lymphocytes (positive for the CD5 pan-T-cell antigen recognized by anti-Leu1).

### *Correlation between diagnosis and the presence of BE2-positive lymphocytes in cutaneous biopsies*

Whereas BE2-positive endothelial cells, Langerhans cells and dermal macrophages were present in all the cutaneous biopsies studied, BE2-positive T-lymphocytes were more restricted in their distribution (as assessed by use of the subclass specific double immunofluorescence method) (Table 1).

Among the malignant T-cell lymphomas, BE2 labelled the neoplastic cells in 17 of 28 cases, and labelling was equally frequent in mycosis fungoides, Sézary syndrome and large cell lymphomas (immunoblastic or lymphoblastic) of T-cell origin. BE2-positive T-lymphocytes were found slightly less frequently among premalignant and pseudomalignant conditions, being identified in two of five cases of actinic reticuloid, in four of eight cases of the large plaque or atrophic variant of parapsoriasis and in none of five cases of lymphomatoid papulosis. However, BE2-positive T cells were clearly not restricted to malignant or premalignant cutaneous T-cell infiltrates, being also found in four of 19 benign dermatoses (Table 1; Fig. 1).

Finally, it should be noted that BE2 labelled cutaneous tumours of non-T-cell origin, including two B-cell neoplasms, a true histiocytic malignancy and a case of histiocytosis X.

### *Reactivity of BE2 with lymphoid organs*

The range of reactivity of BE2 found in cutaneous biopsies was extended by immunostaining normal thymus, hyperplastic tonsil, lymph node and spleen using the same series of staining techniques described above. BE2 labelled the majority of cell types present in these organs, including B-lymphocytes in mantle and marginal zones (Fig. 2), and T-lymphocytes in the paracortex and in the thymic medullary region (Figs 3 and 4), as well as endothelial cells and macrophages present in the red pulp, paracortex and germinal centres (Figs 2 and 3). For lymph nodes, identical staining patterns were seen in follicular hyperplasia, dermatopathic lymphadenitis and nodular T-zone hyperplasia.

The reactivity of BE2 with nodal lymphoid malignancies (Table 3) was in accordance with the labelling found in the normal lymphoid organs, and indicated that BE2 reacted with both B-cell and T-cell lymphomas of widely varying histological subtypes.

## DISCUSSION

It has been proposed previously that antibody BE2, raised against T-cell leukaemia cells and recognizing a 78 kd antigen, reacts specifically with malignant lymphoid cells and is therefore a valuable reagent for the early identification of cutaneous T-cell lymphomas (Berger *et al.*, 1982). Although this concept was primarily based on data obtained by examining blood lymphocytes and cell lines, a limited number of tissue sections including skin, lymph node and thymus were also investigated by two-stage immunofluorescence or immunoperoxidase methods. Whereas BE2 labelled skin and lymph nodes infiltrated by cutaneous T-cell lymphomas, the normal organs were reported to be BE2-negative (Berger *et al.*, 1982).

These data are in striking contrast to the results obtained in the present investigation in which BE2 labelled a number of normal cell types in cutaneous biopsies and lymphoid organs, including B-lymphocytes, T-lymphocytes, macrophages, Langerhans cells and endothelial

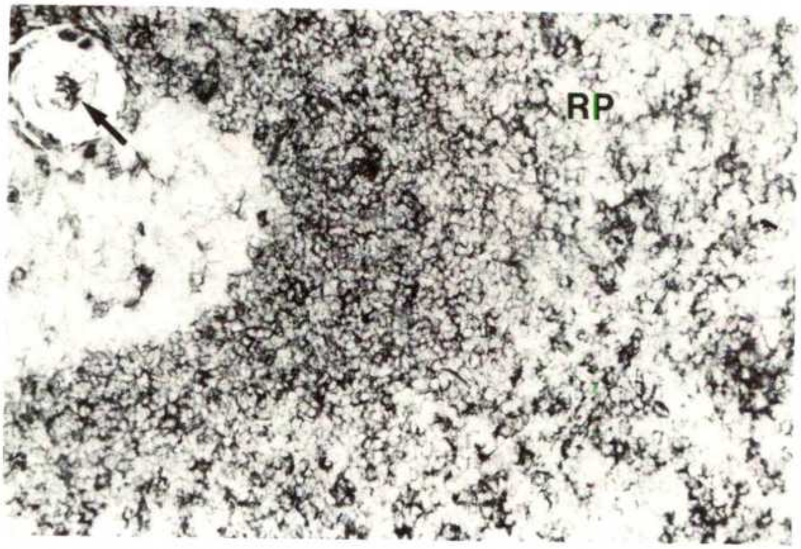

FIGURE 2. BE2-positive B cells in mantle and marginal zones of a benign hyperplastic spleen. Note also the strong reactivity of BE2 with endothelial cells (arrow) and macrophages present in the red pulp (RP). (Original × 400).

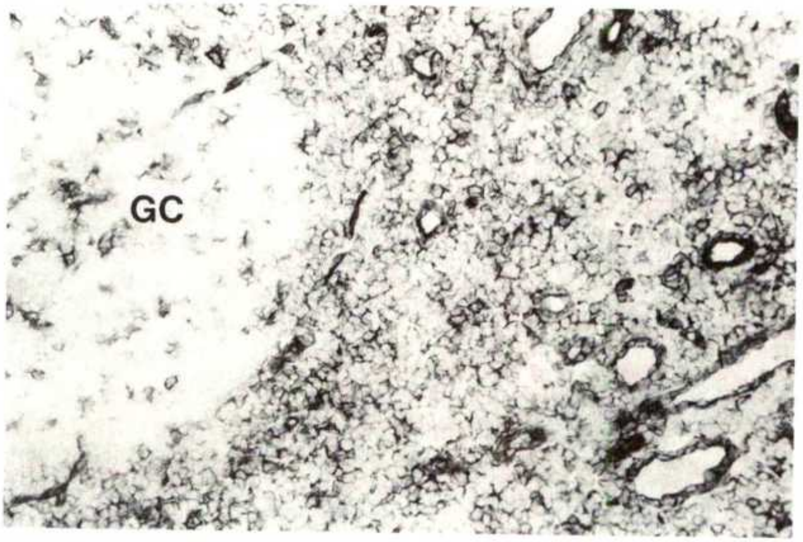

FIGURE 3. BE2-positive paracortical T cells in hyperplastic tonsil tissue. Macrophages in the germinal centre (GC) and endothelial cells are also BE2-positive. (Original × 400).

cells. Confirmation of the lineage of the BE2-positive B-cells and endothelial cells was achieved easily, owing to their distinctive tissue distribution and morphology; while a definite discrimination between BE2-positive macrophages, Langerhans cells and T-lymphocytes usually required the assessment of serial or double stained sections.

At present no definite explanation for the marked discrepancies between the present study and the previous reports can be provided. However, we presume that they are most likely to

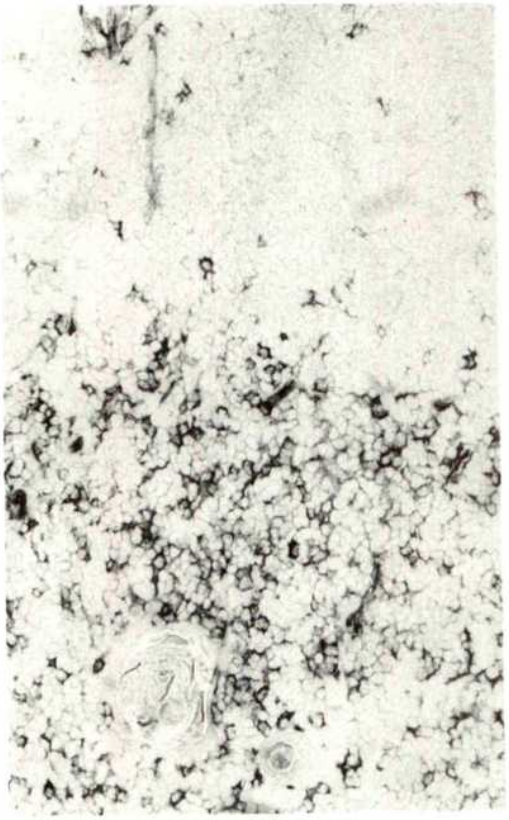

FIGURE 4. BE2-positive medullary T cells in normal thymus. (Original × 400).

TABLE 3. Reactivity of BE2 with nodal lymphoid malignancies

| Phenotype | Histology | BE2-positive | BE2-negative |
|---|---|---|---|
| B-cell type | Chronic lymphocytic leukaemia | 0 | 2 |
| | Centroblastic/centrocytic | 6 | 4 |
| | Centroblastic | 0 | 1 |
| | Immunoblastic | 1 | 1 |
| | Hairy cell leukaemia | 1 | 0 |
| T-cell type | T-zone lymphoma | 3 | 2 |
| | Immunoblastic | 5 | 1 |
| | Lymphoblastic | 2 | 2 |

reflect differences in the sensitivity of the staining procedures adopted. Thus, the multi-stage immunoenzymatic methods employed in the present investigation increase significantly the reliability of identification of antigens in tissue sections (Stein *et al.*, 1984) when compared with the two-stage staining techniques used by Berger *et al.* (1982). The concept that the threshold for detection of BE2 antigen expression is a reflection of the sensitivity of the staining procedure was also supported in the present study by the finding that BE2 labelling by a two-stage method required use of more concentrated antibody than the multi-stage immunoenzymatic staining techniques.

We conclude that BE2 is not specific for malignant lymphoid cells and that use of this reagent on tissue sections is unlikely to improve the diagnosis of cutaneous lymphomas. Whether tumour cell specific antigens do exist within the lymphoid system will be an important issue to elucidate in future research. At present it appears that other approaches, for example DNA flow cytometry (Wantzin *et al.*, 1984) and T-cell receptor gene rearrangement studies (Weiss *et al.*, 1985), may be more likely to provide objective criteria for discrimination between benign and malignant cutaneous lymphoid cell populations.

## ACKNOWLEDGMENTS

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# Apparent progression of lentigo maligna to invasive melanoma during treatment with topical azelaic acid

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## SUMMARY

Nine patients with lentigo maligna were treated with topical azelaic acid. Clinical improvement was observed in four, with complete clearing in one. Two patients developed invasive lentigo maligna melanoma while on treatment. Caution should be exercised in the use of topical azelaic acid in the treatment of lentigo maligna.

Azelaic acid has been used topically in the treatment of more than 51 cases of lentigo maligna in the past 7 years (Breathnach, Nazzaro-Porro & Passi, 1984) and clinical and histological resolution of the lesions were reported in all cases. In the past 4 years, we have treated nine cases of lentigo maligna with topical azelaic acid.

## METHODS

Nine patients (aged 52–82 years) with lentigo maligna confirmed by biopsy, took part in the study. Following biopsy, all patients had a residual macular lesion adjacent to the biopsy site which was clinically consistent with residual lentigo maligna. All lesions were on the face.

The azelaic acid preparations used initially on each patient ranged in concentration from 15% to 35% azelaic acid in a cream (20%) or ointment (15%, 30%, 35%) base (Table 1). The 20% cream was prepared by Schering A.G. and the 35% ointment by Dr M. Nazzaro-Porro. The 15% and 30% ointments were prepared in petrolatum alba. All patients were instructed to apply the cream twice daily to the lesion. The cream was applied quite generously and was rubbed in well. The patients were well motivated and patient compliance appeared to be excellent.

## RESULTS

The results of the treatment are summarized in Table 1. Most patients initially developed

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TABLE 1. Clinical details of nine patients receiving azelaic acid therapy

| Patient no. | Age (years) | Previous therapy | Azelaic acid concentration | Duration of therapy (weeks) | Clinical result of therapy | Biopsy at completion of therapy |
|---|---|---|---|---|---|---|
| 1 | 61 | None | 35% | 16 | Complete clearing | Normal |
| 2 | 72 | None | 15% | 64 | Partial lightening | LM |
| 3 | 78 | None | 15% | 18 | Partial lightening | LM |
| | | | 30% | 32 | Partial lightening<br>Partial darkening | LM |
| | | | 20% | 4 | Partial lightening<br>Partial darkening | LM |
| 4 | 82 | None | 20% | 6 | No change | Not done |
| | | | 35% | 27 | Partial lightening | Not done |
| 5 | 82 | None | 15% | 26 | No change | Not done |
| 6 | 77 | None | 15% | 37 | No change | LM |
| | | | 30% | 8 | No change | LM |
| 7 | 75 | Shave removal × 3 | 15% | 16 | No change | LM |
| | | | 30% | 48 | No change | LM |
| 8 | 56 | Shave removal | 15% | 12 | No change | LMM with minimal invasion |
| 9 | 52 | None | 35% | 10 | No change | |
| | | | 20% | 13 | Nodule developed | LMM invading to 0·4 mm |

LM = lentigo maligna.
LMM = lentigo maligna melanoma (invasive).

erythema at the site of application of the topical azeleic acid, including the patients who progressed to invasive melanoma. This irritation did not prevent any patient from continuing the therapy.

Patient 1 experienced a very rapid lightening of her lesion and the lesion cleared completely both clinically and histologically. Three of the remaining patients showed a partial response with considerable lightening of the lesions. Figure 1 shows the lesion in Patient 3 prior to treatment. Figure 2 shows the same patient after 54 weeks of therapy. There was a partial clearing of some of the peripheral hyperpigmentation, but centrally progressive darkening was noted. Histological examination of this central area showed typical lentigo maligna.

Five patients showed no improvement clinically. Two of these five patients progressed to invasive malignant melanoma, despite continued therapy with azelaic acid. Patient 8 developed invasive melanoma after 12 weeks of the application of a 15% azelaic acid cream. This was an early invasive melanoma, with only a few areas of melanocyte penetration through the basement membrane. Patient 9 progressed to clinically obvious invasive malignant melanoma while on treatment. This treatment consisted of 10 weeks' application of a 35% ointment and 13 weeks application of a 20% azelaic acid cream. At the start of treatment the lesion was completely flat, and it remained so until the final 4 weeks of therapy when a nodule developed within it. Biopsy of this nodule showed a lentigo maligna melanoma invading to 0·4 mm (Fig. 3).

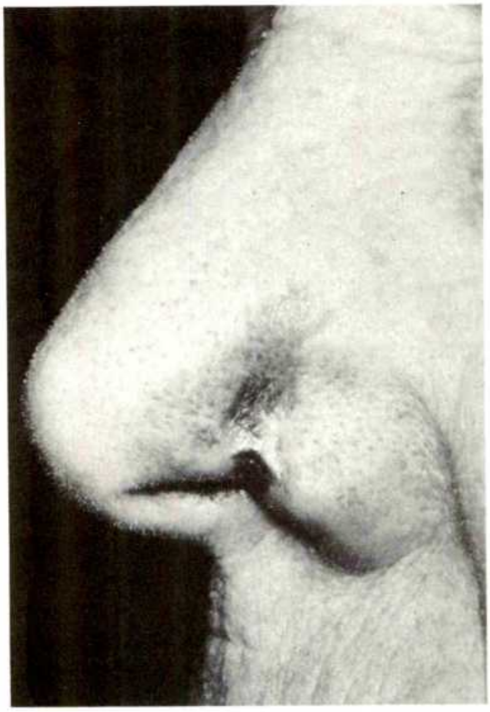

FIGURE 1. Patient 3. After biopsy, prior to therapy.

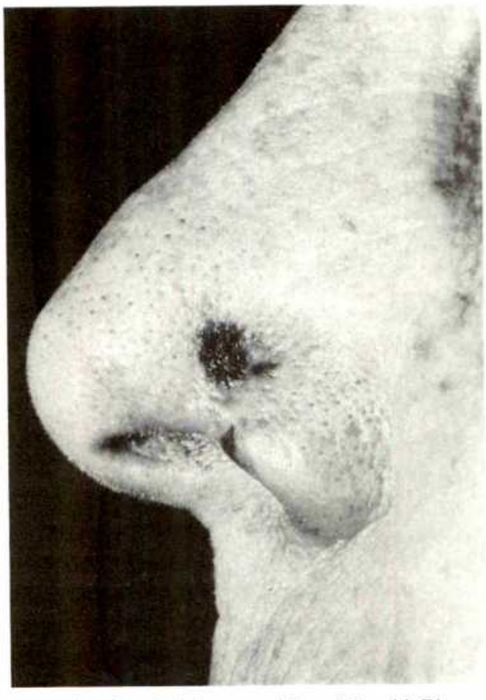

FIGURE 2. Patient 3. After 54 weeks of topical therapy with azelaic acid. Biopsy of central hyperpigmented area showed residual lentigo maligna.

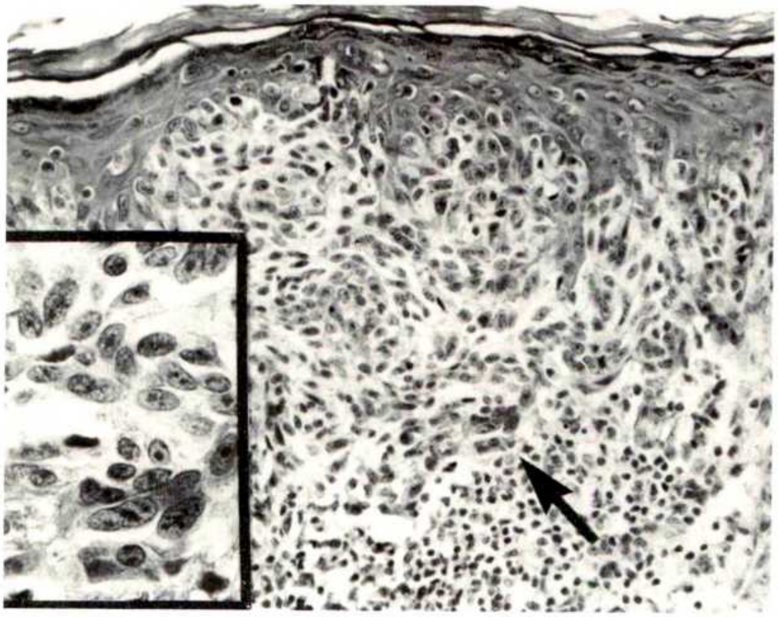

FIGURE 3. Patient 9. Lentigo maligna melanoma invading to 0·4 mm (Breslow) which developed during azelaic acid therapy. (Original × 240; inset (arrow) × 600).

## DISCUSSION

This report confirms the ability of topical azelaic acid to produce a clinical response when used to treat lentigo maligna. Four patients showed a clinical improvement, and one patient cleared completely. This response accords well with the published reports of the efficacy of azelaic acid in the treatment of lentigo maligna (Breathnach *et al.*, 1984; Nazzaro-Porro *et al.*, 1979; Leibl *et al.*, 1985) and superficial spreading and nodular melanoma (Nazzaro-Porro *et al.*, 1980) in man. In mice, Harding-Passey melanoma appears to respond to azelaic acid (Nazzaro-Porro *et al.*, 1978) but B16 melanoma does not (Pathak *et al.*, 1979). Apart from stimulated melanogenesis, normal melanocytes are apparently not significantly affected by azelaic acid (Breathnach *et al.*, 1979).

Five of our patients failed to demonstrate a beneficial response to topical azelaic acid. While four of them had started on the 15% ointment, this concentration has been reported previously to be effective (Nazzaro-Porro *et al.*, 1979). Three of the five eventually used a 30% or 35% ointment. The lack of response in these five patients could have been due to poor patient compliance, but there is no evidence for this. All five patients developed a typical irritant erythematous reaction to azelaic acid, indicating that they were indeed using the agent. Poor penetration may also have been a factor. Although the 15% ointment was prepared in our pharmacy, Patient 9, who progressed to significantly invasive melanoma, was using the same 35% ointment and 20% cream which have been successful in other patients (Breathnach *et al.*, 1984).

It is possible that a longer duration of therapy would have produced a more complete response in some patients. Patient 3 was treated for 54 weeks, during which time there was continued expansion of central hyperpigmentation. It is unlikely that Patient 9 would have benefited from longer treatment, in view of the rapid growth of an invasive melanoma while on treatment.

Another explanation of a lack of response is that some lentigo maligna cells do not respond to azelaic acid. This is the most likely. The variable response of the lesion in Patient 3 would support this concept, as part of the lesion faded, while another area apparently progressed.

Further study of azelaic acid is certainly indicated. This agent appears to have significant promise in the treatment of premalignant melanocytic skin lesions, and possibly even invasive malignant melanoma. However, because of the possibility of some patients progressing to invasive malignant melanoma while on therapy, considerable caution must be exercised. Indeed, it should be used only in an investigational setting with the patient being closely monitored. Patients on treatment should be aware of the possibility of tumour progression.

Breathnach *et al.* (1984) have stated that azelaic acid is 'a most satisfactory form of therapy, especially in early cases, and for extensive long-standing lesions in older patients'. We would suggest that the first line of treatment of lentigo maligna remains surgical excision, and that, at present, azelaic acid should be used only in an investigational setting and only when alternative proven forms of therapy are not possible.

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# Aneuploidy in Bowen's disease

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## SUMMARY

Distinct aneuploid clones of keratinocytes were detected in six cases of cutaneous Bowen's disease. As Bowen's disease is an accepted precursor of malignancy, aneuploidy may prove to be a useful indicator of the risk of invasive change in dermatoses questionably pre-malignant.

'Aneuploidy', the possession of abnormal amounts of cellular DNA, is a feature of many human tumours. For example, Holm (1982) reported this in 75% of squamous cell carcinomas of the head and neck. We used flow cytometry (FCM) to detect aneuploidy in a pre-malignant dermatosis, namely Bowen's disease, in order to investigate the use of aneuploidy as a predictive index of malignant change in skin disease.

## METHODS

Six lesions clinically diagnosed as Bowen's disease were biopsied from five patients (four female, one male). Four lesions were on the lower leg, one was vulval, and one was on the hand. All six lesions were longstanding, having been present for 1–7 years, and were fixed plaques typical of Bowen's disease. Four patients had had cutaneous squamous cell carcinomas previously at other sites, or had such lesions at the time of biopsy. None of these patients was receiving any drugs or treatment with UVB or psoralen and UVA (PUVA).

An ellipse of skin, 8 mm long, was removed from each lesion under local anaesthesia, and was bisected longitudinally. One half was fixed in formalin, and sections were stained with haematoxylin and eosin for histological examination. The degree of epidermal dysplasia was graded by one of us (D.H.M.) on a scale from 1 to 10. The criteria used were loss of cellular polarity, nuclear size, multinucleated keratinocytes, apoptotic cells in the epidermis and atypical mitoses in the epidermis.

The other half of the specimen was placed in Eagle's minimal essential medium (MEM) containing antibiotics. This sample was then processed for FCM within 24 h. Tissue was incubated in collagenase and dithioerythritol for 1 h to split the epidermis from the dermis. The

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epidermal cells were then disaggregated using trypsin and the resultant keratinocyte suspension was stained with 2,4,diamidino,2,phenylindole dihydrochloride, (DAPI) (Boehringer Mannheim, West Germany). $10^5$ cells were run on a Becton Dickinson FACS Analyser using a peak excitation wavelength of 360 nm, and collecting fluorescent light at 490 nm. Each specimen was run with a suspension of chicken red blood cells as an internal DNA standard. Aneuploidy was defined as the presence of a clone of cells producing an FCM peak quite distinct from the normal G1 peak.

## RESULTS

### *Flow cytometry*

All six specimens showed distinct aneuploid clones of cells. The results are summarized in Table 1, and a typical tracing from Patient 5 is shown in Figure 1. In Table 1, the results are expressed as the DNA index of each sample. This figure indicates the quantity of DNA of any aneuploid peak in relation to that of the diploid G1 peak. A DNA index of 1·0 would therefore signify the presence of only diploid cells.

TABLE 1. History, histology and flow cytometry results in five patients with Bowen's disease

| Patient number | Ploidy | DNA index | Coefficient of variation of $G_1$ peak (%) | Histology grade | Past history |
|---|---|---|---|---|---|
| 1 specimen (a) | Aneuploid | 1·4 | 3·9 | 2 | SCC leg |
| specimen (b) | Aneuploid | 1·4 | 6·5 | 4 | — |
| 2 | Aneuploid | 1·7 | 4·3 | 7 | SCC leg |
| 3 | Aneuploid | 1·2 | 5·6 | 9 | SCC vulva |
| 4 | Aneuploid | 1·3 | 3·6 | 9 | SCC in large Bowen's plaque |
| 5 | Aneuploid | 1·3 | 4·6 | 3 | — |

SCC = squamous cell carcinoma.

### *Histology*

Histology confirmed Bowen's disease in all six specimens. The lesions showed degrees of dysplasia ranging from 2 to 9 on our grading scale. Thus, aneuploidy was a feature of histologically mildly dysplastic Bowen's disease, as well as of grossly dysplastic lesions.

## DISCUSSION

Aneuploidy is a feature of the majority of human solid tumours. Barlogie (1984) quoted a frequency of 75% for all solid tumours. When present, an abnormal DNA stemline, or a clone of cells in a pre-cancerous condition, is thus held to imply neoplasia (Barlogie, 1984). For example, such aneuploid clones have been demonstrated, although not consistently, in atrophic chronic gastritis (Teodori *et al.*, 1984). However, the prognostic implications are, as yet, unknown,

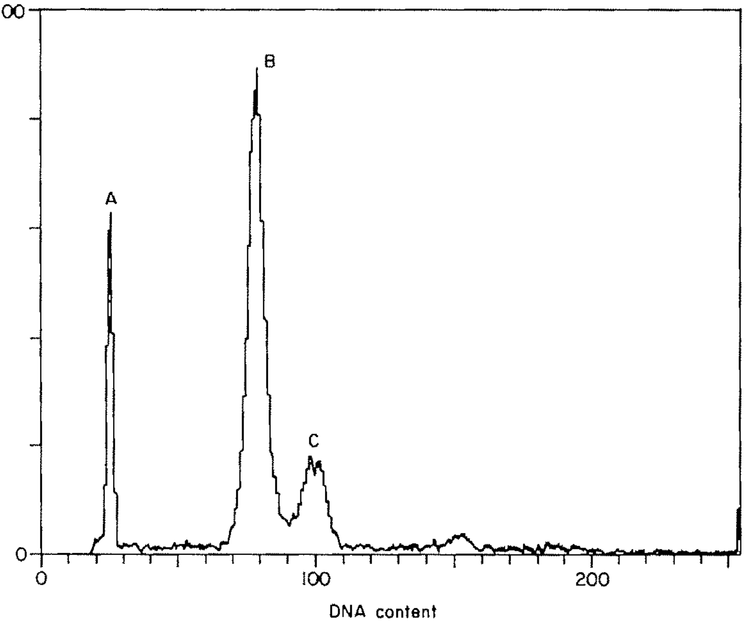

FIGURE 1. A typical tracing from Patient 5 showing distinct aneuploid peak with a DNA index of 1 3. The X axis represents relative DNA content and the Y axis number of cells.
A: Chicken red blood cells run as an internal DNA standard.
B: Diploid $G_1$ peak.
C. Aneuploid $G_1$ peak.

although some authors have claimed that the development of aneuploidy can parallel disease progression (Lange Wantzin *et al.*, 1985).

The malignant potential of many skin diseases, for example lichen sclerosus et atrophicus, is questionable. In an attempt to determine whether aneuploidy can be used to predict malignant change, we chose to investigate aneuploidy in Bowen's disease, as invasive squamous carcinoma is said to occur in about 5% of cases (Graham & Helwig, 1961).

Distinct aneuploid clones of cells were demonstrated in six lesions of Bowen's disease. A previous study of seven cases, (Frentz & Moller, 1983) failed to demonstrate convincing evidence of aneuploidy. These authors demonstrated a broad G1 peak in four of their seven cases, and have classified this as aneuploidy. The coefficient of variation (CV) of the G1 peak values for normal skin in that study ranged from 6% to 14%. In our hands, the mean CV of $G_1$ peak values for six samples of normal skin was 3·8%. Similarly, the CV of tumour peaks in Frentz and Moller's study ranged from 6% to 19%. In our study, the mean CV of G1 peaks of Bowen's lesions was 4·9%. We feel that, as stated by Bauer *et al.* (1985), numerous technical factors can influence the width of $G_1$ peaks, and therefore that there are considerable dangers in making the assumption that broad $G_1$ peaks necessarily equate with aneuploidy. In the present study, in which the quality of results was good, as demonstrated by the relatively low CVs, it was possible in all six lesions studied to demonstrate distinct aneuploid peaks (Fig. 1).

Attempts were made to correlate aneuploidy with the degree of histological atypia. It was found that aneuploidy occurred in mildly atypical lesions as well as in grossly atypical lesions.

A correlation was also sought between the clinical status of the patient and the FCM results.

All our cases were frankly aneuploid, whilst the incidence of invasive change in Bowen's disease is said to be only 5%, so the presence of aneuploidy is unlikely to have prognostic implications for individual lesions of Bowen's disease.

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***Pharmacology and Treatment***

# Seborrhoeic dermatitis and *Pityrosporum orbiculare*: treatment of seborrhoeic dermatitis of the scalp with miconazole-hydrocortisone (Daktacort), miconazole and hydrocortisone

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## SUMMARY

Seventy patients (36 males and 34 females) with seborrhoeic dermatitis of the scalp were treated in a double-blind controlled study, for a maximum of 6 weeks, with 2% miconazole base and 1% hydrocortisone (Daktacort), 2% miconazole base, or 1% hydrocortisone. Patients who were cured were treated with the same formulation prophylactically twice monthly for 3 months or until recurrence. Nineteen of 21 patients were cured in the Daktacort group, 15 of 22 in the miconazole group and 17 of 24 in the hydrocortisone group. The number of cultured *Pityrosporum orbiculare* was significantly lower in all groups after treatment, but in the hydrocortisone group was still significantly higher than in the two other groups. After 3 months of prophylactic treatment, both Daktacort (16 of 19 patients clear) and miconazole (10 of 15 patients clear) were significantly better than hydrocortisone (3 of 17 patients clear) ($P < 0{\cdot}01$). The numbers of *P. orbiculare* remained low in the Daktacort and miconazole groups and also significantly lower than in the hydrocortisone-treated group ($P < 0{\cdot}01$). In patients with recurrence, the numbers returned to pre-treatment levels. This study demonstrates the aetiological significance of the *Pityrosporum* yeasts in seborrhoeic dermatitis. Both Daktacort and miconazole were effective in treatment and as prophylactic agents.

Seborrhoeic dermatitis of the scalp is characterized by yellowish adherent greasy scales. Dandruff is a scaly condition of the scalp without evidence of other skin diseases. It is almost impossible to make a clear-cut division between dandruff and mild seborrhoeic dermatitis. The exact aetiology of seborrhoeic dermatitis and dandruff is still unclear. Although Kligman and his co-workers concluded that dandruff and seborrhoeic dermatitis were not caused by the *Pityrosporum* yeasts (McGinley *et al.*, 1975; Kligman, McGinley & Leyden, 1979), many studies now link the yeast with the disease (Alexander, 1967; Gosse & Vanderwyk, 1969; Rosenberg, Belew & Skinner, 1984; Ford *et al.*, 1984; Shuster, 1984). Many antimycotics have been effective in treatment (Ford *et al.*, 1984; Gosse & Vanderwyk, 1969), and cure of seborrhoeic dermatitis is often paralleled by a fall in the numbers of *P. ovale* (*P. orbiculare*) (McGinley *et al.*, 1975; Gosse & Vanderwyk, 1969; Skinner *et al.*, 1985).

Although many studies have shown an effect of antifungals in the treatment of dandruff and seborrhoeic dermatitis, most of them were open, involving small numbers of patients. Double-blind controlled studies are now needed. We report here the results of a double-blind controlled trial to investigate the effectiveness of miconazole plus hydrocortisone (Daktacort), compared with miconazole alone or hydrocortisone alone, in the treatment of seborrhoeic dermatitis of the scalp.

## METHODS

### *Patients*

Seventy patients (36 males and 34 females, aged 21–69 years, mean 38 years) with seborrhoeic dermatitis of the scalp were included in the study. All patients had positive cultures for *P. orbiculare* before entering the study. No topical or systemic treatment with antifungal agents or corticosteroids was permitted for 3 weeks prior to the start of the study. The patients were instructed not to shampoo their hair for 5 days prior to each assessment of scalp lesions.

### *Treatment*

The patients were randomly allocated to treatment with 2% miconazole base and 1% hydrocortisone (Daktacort), 2% miconazole base, or 1% hydrocortisone. These drugs were each incorporated in a solution containing 60% ethyl alcohol, 10% propylene glycol, and purified water. Twenty-three patients were treated with Daktacort, 23 with miconazole base, and 24 with hydrocortisone. The solutions were provided by Janssen Pharmaceutica, Helsingborg, Sweden.

The solution was applied to the whole scalp every evening for 3 weeks. Patients were seen 5 days after the last day of treatment, and if they were not considered cured, the treatment was continued for an additional 3 weeks. When a patient was considered cured he was given the same solution as he had already been using, (still double-blind), to apply twice monthly for 3 months or until recurrence of the seborrhoeic dermatitis.

Patients were assessed before treatment and at follow-up visits 5 days after finishing treatment, and monthly thereafter.

### *Assessment of lesions*

Lesions were examined and scored on a quadrant-area-severity scale. The scalp was divided into four quadrants. The area of involvement of each quadrant was measured on a 1–5 scale where 1 indicated less than 10% involvement and 5 more than 70% involvement. Severity was measured by a 0–3 scale where 0 indicated healed and 3 erythema with thick confluent plates of yellow-white scales. The total scalp score was then obtained by multiplying the total area of involvement by total severity score.

### *Quantitative culture of* Pityrosporum orbiculare

Scalp washings were obtained and diluted in 0·075 M phosphate buffer pH 7·9 containing 0·1% Triton X-100, as described previously (Faergemann, 1983). Samples (0·1 ml) from the dilutions were inoculated onto glucose-neopeptone-yeast extract agar containing olive oil (2%), Tween 80 (0·2%) and glycerol monostearate (2·5 g/l) (Faergemann & Bernander, 1981). Plates were incubated at 37°C and examined after 6 days. Specimens were taken from the vertex area of the scalp and transferred to the culture medium within $\frac{1}{2}$ h.

### *Statistics*

Scalp scores and the numbers of *P. orbiculare*, before and after treatment, were compared using Student's $t$-test for paired samples, and between treatments using an unpaired $t$-test. The number of patients cured, the number of treatment failures, the number of patients who were still free from lesions after 3 months of prophylaxis, and the number of patients who relapsed were compared using the chi-squared test with Yates' correction.

## RESULTS

Three patients, two in the Daktacort group and one in the miconazole base group did not return for follow-up.

The results of treatment are shown in Table 1. All three formulations were effective, with cure rates of 68%–91%. There were no significant differences in the number of weeks necessary to obtain a cure in the three groups (chi-squared test). In the Daktacort group nine patients were cured after 3 weeks; in the miconazole base group, five were cured after 3 weeks; and in the hydrocortisone group eight were cured after 3 weeks of treatment.

The numbers of *P. orbiculare* cultured were reduced significantly with all treatments (Table 2). The largest reductions in the number of organisms were found after treatment with Daktacort and with miconazole base. Before treatment there were no significant differences in counts between the three groups, but after treatment the counts were significantly lower in the Daktacort and the miconazole base groups compared with the hydrocortisone group ($P < 0{\cdot}01$). For Daktacort and miconazole base the counts for *P. orbiculare* were also significantly lower after treatment in the treatment failures ($P < 0{\cdot}01$).

The results of prophylaxis are given in Table 3. After 3 months of prophylactic treatment the results with both Daktacort (84% of patients clear) and miconazole base (67% clear) were significantly better than with hydrocortisone (18% clear) ($P < 0{\cdot}01$). The number of cultured

TABLE 1. Results of treatment of seborrhoeic dermatitis of the scalp

| | Number of patients (mean lesion score ± SD) | | |
|---|---|---|---|
| | Daktacort | miconazole base | hydrocortisone |
| Before treatment | 23 (37 ± 12) | 23 (37 ± 10) | 24 (37 ± 10) |
| After treatment: | | | |
| Cured* | 19 (0 ± 1) | 15 (0 ± 1) | 17 (0 ± 1) |
| Treatment failures | 2 (20 ± 6) | 7 (14 ± 9) | 7 (32 ± 32) |
| Dropped out | 2 | 1 | 0 |

* No significant difference between the numbers of patients cured in the three groups.

TABLE 2. Numbers of *Pityrosporum orbiculare* cultured from the scalp of patients with seborrhoeic dermatitis before and after treatment

| | Number of organisms/cm² (mean ± SD) | | |
|---|---|---|---|
| | Daktacort | miconazole base | hydrocortisone |
| Before treatment | 2067 ± 1997 | 1767 ± 1853 | 1540 ± 1145 |
| After treatment: | | | |
| Cured* | 19 ± 25 | 13 ± 20 | 512 ± 317 |
| Treatment failures | 210 ± 297 | 50 ± 40 | 743 ± 280 |

* With all three treatment regimens the numbers of organisms cultured after treatment were significantly lower than before treatment ($P < 0.01$).

TABLE 3. Results of prophylactic treatment of seborrhoeic dermatitis of the scalp

| | Number of patients (mean lesion score ± SD) | | |
|---|---|---|---|
| | Daktacort | miconazole base | hydrocortisone |
| Free from lesions* | 16 (0) | 10 (0) | 3 (0) |
| Recurrence | 3 (32 ± 8) | 5 (27 ± 6) | 14 (28 ± 13) |

* Daktacort and miconazole base were significantly better than hydrocortisone ($P < 0{\cdot}01$). There was no significant difference between Daktacort and miconazole base ($P > 0{\cdot}05$).

TABLE 4. Number of *Pityrosporum orbiculare*/cm² cultured from the scalp in patients on prophylactic treatment of seborrhoeic dermatitis

| | Number of organisms/cm² (mean ± SD) | | |
|---|---|---|---|
| | Daktacort | miconazole base | hydrocortisone |
| Patients without lesions* | 14 ± 26 | 20 ± 25 | 327 ± 261 |
| Patients with recurrence | 753 ± 926 | 1064 ± 1711 | 1418 ± 905 |

* Numbers of organisms cultured from patients without lesions were significantly lower than in patients with recurrence ($p < 0.01$).

*P. orbiculare*/cm² remained low in patients without recurrence treated with Daktacort and with miconazole base (Table 4). In the hydrocortisone group without recurrence the number was still lower than before treatment but significantly higher than in the Daktacort and miconazole base groups $P < 0{\cdot}01$. There was no statistically significant difference in the numbers of organisms cultured from patients with recurrence compared with the numbers found before treatment.

## DISCUSSION

*P. orbiculare* is not only a member of the normal human cutaneous flora but also an opportunistic pathogen. The role of the *Pityrosporum* yeasts in pityriasis versicolor and pityrosporum folliculitis is now well established (Faergemann & Fredriksson, 1981; Ford, Ive & Midgley, 1982; Bäck, Faergemann & Hörnquist, 1985). There are now several reports indicating a link between the *Pityrosporum* yeasts and seborrhoeic dermatitis. The highest antibody titres against *P. orbiculare* and *P. ovale* have been found in patients with dandruff and seborrhoeic dermatitis (Alexander, 1968). The *Pityrosporum* yeasts activate the alternative complement pathway (Sohnle & Collins-Lech, 1983; Belew, Rosenberg & Jennings, 1980) and this is a possible explanation for the inflammation seen in seborrhoeic dermatitis. They also have a lipase activity (Wilde & Stewart, 1968; Marples, Downing & Kligman, 1972; Porro *et al.*, 1976) and the generation of free fatty acids may also partially explain the inflammation. Experimental seborrhoeic dermatitis-like lesions have been produced with *Pityrosporum* yeasts in guinea-pigs (Drouhet *et al.*,1980).

Cure of seborrheic dermatitis and dandruff is often paralleled by a fall in the numbers of *Pityrosporum* yeasts (McGinley *et al.*, 1975; Alexander, 1967; Gosse & Vanderwyk, 1969). Many antimycotics have been found to be effective in earlier studies on small numbers of patients (Gosse & Vanderwyk, 1969; Ford *et al.*, 1984). In addition, Gosse and Vanderwyk (1969) showed that re-infection of the scalp with a nystatin-resistant *P. ovale* strain induces a relapse of dandruff.

In the present double-blind controlled study, Daktacort, miconazole base and hydrocortisone were all effective in the treatment of seborrhoeic dermatitis of the scalp; Daktacort giving the highest cure rate (91%). Effective treatment was paralleled by a significant fall in numbers of *P. orbiculare*/cm$^2$. This was seen also with hydrocortisone, but the fall in numbers was significantly less than in the Daktacort and the miconazole base treated groups. The smaller numbers of *P. orbiculare* seen in the hydrocortisone group after successful treatment may be explained partially by the smaller surface area of the scalp in cured patients. Also the solutions contained 10% propylene glycol and 60% isopropyl alcohol, both of which have antimycotic activity (Faergemann & Bernander, 1979; Faergemann & Fredriksson, 1980).

Both Daktacort and miconazole base were significantly better than hydrocortisone as prophylactic agents. Here the higher antimycotic activity of miconazole base compared with the base solution is probably important. Recurrence of seborrhoeic dermatitis was paralleled by a rise in numbers of *P. orbiculare*. There were no significant differences in the numbers of organisms cultured in patients with recurrence compared with the numbers seen before treatment. The largest number in patients with recurrence was seen in the hydrocortisone group.

This study demonstrates clearly the aetiological significance of the *Pityrosporum* yeasts in seborrhoeic dermatitis of the scalp. There is a parallel between the presence of the yeasts and the disease. Although patients can be cured with a pure hydrocortisone solution, the relapse rate, even with twice monthly prophylactic treatment, is very high. Both Daktacort and miconazole base were very effective in treatment of seborrhoeic dermatitis of the scalp and very effective as prophylactic agents when used twice monthly. Further studies are needed to test various different treatment and prophylaxis schedules.

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# Itraconazole, a new orally active antifungal, in the treatment of pityriasis versicolor

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## SUMMARY

Itraconazole, a new orally active triazole antifungal, has been tested in patients with pityriasis versicolor. A comparison of different dose schedules was carried out in 73 patients. A regime of 100 mg itraconazole daily for 15 days gave a 100% response rate; 200 mg daily for 5 days gave an 80% response rate. Two patients, who had received 50 mg itraconazole for 14 days, relapsed within 2 months of finishing treatment. Only two patients reported minor side-effects.

Itraconazole (R 51211) is a triazole derivative which has *in vitro* and *in vivo* activity against a wide range of human pathogenic fungi (van Cutsem *et al.*, 1983). Animal studies have indicated that itraconazole is 5–10 times more potent than ketoconazole under identical conditions (van Cutsem & Janssen, 1984). Itraconazole also shows a marked cidal effect on *Pityrosporum ovale* and *P. orbiculare*, the causative organisms of pityriasis versicolor (van Cutsem & Janssen, 1985).

Recent work with oral ketoconazole has established that short-term therapies can be used for treatment of pityriasis versicolor (Borelli, 1980). The aim of the present study was to determine an optimal treatment regimen using the drug in a polyethylene glycol (PEG) formulation.

## METHODS

Patients with microscopically proven pityriasis versicolor and a positive Wood's light test were included in the trial. Women who were pregnant or who were not using adequate birth control methods were excluded. Use of other oral or topical antifungals, including selenium sulphide, was stopped one month prior to commencement of the trial.

The following treatment schedules were tested, 50 mg capsules being given throughout; 50 mg/day for 14 days; 100 mg/day for 5, 10 or 15 days and 200 mg/day for 5 days. The medication was always taken with a meal.

Patients were seen for clinical and mycological evaluation immediately before starting the treatment, one week after the end of the treatment and attended for follow up 2 months after the

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second evaluation. At each visit, objective and subjective signs and symptoms were scored as severe, marked, moderate or absent. Wood's light examination and microscopy were recorded as positive or negative, and patients were questioned about side-effects.

## RESULTS

Seventy-three patients (35 male, 38 female) were included in the study; 41 had relapsed after previous antifungal therapy and 32 were undergoing treatment for the first time. Seventeen patients received 50 mg itraconazole daily for 14 days, 12 received 100 mg daily for 5 days, 12 received 100 mg daily for 10 days, 12 received 100 mg daily for 15 days and 20 received 200 mg daily for 5 days.

The outcome of treatment was rated as: cure (clinically and mycologically negative), marked improvement (minor residual lesions present, irrespective of the mycological result), and no response. The response rate was taken as the total number of patients cured or markedly improved. The overall results are presented in Table 1.

TABLE 1. Responses of 73 patients with pityriasis vesicolor to oral itraconazole

| No. of patients showing: | Treatment regimen | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 50 mg 14 days | | 100 mg 5 days | | 100 mg 10 days | | 200 mg 5 days | | 100 mg 15 days | |
| Cure | 8 | 11 (65%) | 7 | 7 (64%) | 7 | 7 (58%) | 14 | 12 (85%) | 12 | 12 (100%) |
| Marked improvement | 3 | | 0 | | 0 | | 3 | | 0 | |
| Moderate improvement | 4 | 6 (35%) | 3 | 4 (36%) | 1 | 5 (42%) | 3 | 3 (15%) | 0 | 0 (—) |
| No change | 2 | | 1 | | 4 | | 0 | | 0 | |
| Dropped out | 0 | | 1 | | 0 | | 0 | | 0 | |
| Total | 17 | | 12 | | 12 | | 20 | | 12 | |

In the first group (50 mg/day for 14 days), 11 out of the 17 patients (65%) responded to therapy. The response rates in the groups that had received 100 mg itraconazole for 5 and 10 days respectively were similar (64% and 58% respectively). The 10-day group included two diabetic patients, one of whom was on cimetidine and antacids. Neither patient responded to therapy. In addition, in the group receiving 100 mg for 5 days, one patient who did not respond to treatment had received antacids concomitantly.

The regimen of 200 mg/day for 5 days produced a good response in 17 out of the 20 patients. In the group receiving 100 mg/day for 15 days, all the patients were found to be cured one week after the end of the treatment.

Only 18 patients returned for the 2 month follow-up. Of these, only two patients showed signs of relapse. They had both received 50 mg/day for 14 days. Four patients were seen with a relapse one year after the end of itraconazole treatment (two had received 50 mg/day for 14 days, and two had taken 200 mg/day for 5 days).

No side-effects were reported by the patients receiving 50 mg itraconazole/day for 14 days, 200 mg/day for 5 days or 100 mg/day for 15 days. One patient receiving 100 mg for 5 days, reported nausea and fatigue, and one patient receiving 100 mg/day for 10 days reported nausea.

## DISCUSSION

This study has shown that a short course of itraconazole, in a PEG formulation, appears to be an effective treatment for pityriasis versicolor.

Short courses of ketoconazole have previously been found to be effective in pityriasis versicolor. The initial studies on the use of oral ketoconazole in pityriasis versicolor reported a 98% response rate after a median treatment period of 3–4 weeks (Levine, 1982). More recently, 10 day ketoconazole therapy has been reported to give excellent results (Meisel, 1983).

Acceptable systemic therapy for a relatively benign skin condition such as pityriasis versicolor requires a maximum reduction in the length of therapy compatible with a good response rate. Our present results suggest 200 mg itraconazole daily for 5 days is an effective treatment for pityriasis versicolor. Investigation of other treatment schedules may be of value. Failure of the treatment in patients receiving antacids suggests that itraconazole and antacids should not be given concomitantly. In view of the isolated reports of liver toxicity with long term ketoconazole it seems advisable to carry out liver function tests when new orally active antifungals are investigated. In a recent report on 3 years of clinical experience with itraconazole (Cauwenbergh *et al.*, 1985) abnormal liver function tests were found in eight out of 195 patients (4%), of whom seven were on long term therapy for systemic mycoses; four of them had had abnormal liver function tests at the start of treatment with itraconazole.

The absence, to date, of reports of serious adverse effects is favourable for the continued clinical investigation of itraconazole.

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# Anti-androgen treatment in women with acne: a controlled trial

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## SUMMARY

Ninety female patients with acne were allocated randomly to one of three groups and treated either with Diane, a high dose cyproterone acetate (CPA) regime with ethinyloestradiol, or Minovlar. The same dose of oestrogen was common to all three treatment groups.

Patients were assessed every 2 months for 6 months, by grading for severity of the acne, lesion counts and photography, and subjectively using a visual analogue scale. In addition, bacteriological sampling and sebum excretion rate (SER) measurements were performed.

The results showed a clinical improvement in all three treatment groups, but a more rapid and complete response was seen in those groups who received CPA. There was also a consistent trend suggesting a more favourable response in those in the high dose CPA group. Although there was a marked reduction in SER in the groups treated with CPA, there was no correlation between reduction in SER and clinical improvement in individuals, nor could a reduction in the surface bacterial population be shown to be a primary event in the success of anti-androgen therapy. We have shown that the addition of CPA to oestrogen adds significantly to the therapeutic effect in acne and that anti-androgen and oestrogen combinations are more effective than standard oestrogen and progestagen contraceptive pills.

The aetiology of acne is unknown, although several pathogenic factors are recognized. Sebum production, which is known to be androgen dependent (Strauss, Kligman & Pochi, 1962; Pochi, Strauss & Mescon, 1962; Pochi & Straus, 1969) is of primary importance, and a correlation exists between sebum excretion rate (SER) and the severity of acne (Burton & Shuster, 1971; Pochi & Strauss, 1964; Cunliffe & Shuster, 1961). A 35% reduction in SER is usually associated with a satisfactory inprovement in acne (Cunliffe, 1982). Androgens may also play a part in the disturbed keratinization of the sebaceous follicle infundibulum which is involved in comedo formation (Shuster *et al.*, 1980).

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Treatment with large doses of oestrogens significantly reduces sebaceous gland activity and this is associated with clinical improvement in the acne (Pochi & Strauss, 1973; Strauss & Pochi, 1964). Combined oral contraceptives (O/C) now contain a maximum of 50 μg of oestrogen, but even with this dose, reductions in SER (Pye *et al.*, 1977) and increases in sex hormone binding globulin (SHBG) (Pogmore & Jequier, 1979) can be demonstrated. Oral contraceptives containing less than 50 μg oestrogen, however, have relatively little effect (Pye *et al.*, 1977; Pogmore & Jequier, 1979). The most effective hormonal combination appears to be oestrogen combined with the anti-androgen and progestagen cyproterone acetate (CPA). CPA competes with dihydrotestosterone (DHT) for the peripheral androgen receptor (Schleusener & Neumann, 1981), and inhibits translocation of the hormone receptor complex into the cell nucleus (Bruchovsky, 1980). This combination of an anti-androgen and oestrogen reduces SER by up to 75% (Cunliffe, 1982; Fanta & Muller, 1978; Winkler & Schaefer, 1973) and has been shown to be clinically effective in acne in trials using both high and low doses of CPA (Fanta & Muller, 1978; Hammerstein *et al.*, 1975; van Velde, 1977; Ekoe, Burckhardt & Ruedi, 1980; Aydinlik & Lachnit-Fixson, 1977; Mugglestone & Rhodes, 1982; Breckwoldt *et al.*, 1978; Moltz, Meckies & Hammerstein, 1979; Lachnit-Fixson, 1979).

In the combined O/C's the reduction in the dose of oestrogen has been associated with the introduction of the 19-nor-testosterone derivative progestagens which have a small amount of residual androgenic activity. As a result, acne may be exacerbated, or even provoked, in those not previously affected (Barranco & Jones, 1974; Kirk, 1971). Although both norgestrel and norethisterone have been shown to reduce circulating SHBG levels (El Makhzangy, Wynn & Lawrence, 1979), norethisterone does so to a much lesser extent and Minovlar (norethisterone 1 mg + ethinyloestradiol 50 μg) has been shown to produce a significant rise in SHBG (El Makhzangy *et al.*, 1979; Bowles & Mills, 1981) and a 30% reduction in SER (Pye *et al.*, 1977).

The aim of this trial was to determine in a randomized double-blind study, whether CPA adds significantly to the effectiveness of oestrogen in the treatment of acne, by comparing CPA with a relatively non-androgenic progestagen, norethisterone, with all patients receiving a fixed dose of oestrogen.

## METHODS

### *Patients*

Ninety women aged 16–36 with moderate to severe acne (graded according to Burke & Cunliffe, 1984) were entered into the trial (details are given in Table I). Those with medical contra-indications to the therapy or who continued to smoke more than five cigarettes daily were excluded. Informed consent was obtained from all the patients. All acne medication except the contraceptive pill was stopped for 6 weeks prior to inclusion in the study. Oral contraception was continued until the commencement of the trial.

All assessments were done at the midpoint of the cycle, i.e. between days 10–18 (day one is defined as the first day of menstrual bleeding). The patients had two baseline examinations, separated by one week, before commencement of the trial and were then assessed midway through the second, fourth and sixth cycles of treatment. At the initial visit, they were stratified for severity, using baseline total grades and randomly allocated to one of the following three treatment groups:

*Group M (Minovlar)*. Norethisterone acetate 1 mg and ethinyl oestradiol 50 μg: a combined preparation taken daily from days 5–25 of the cycle inclusive; plus: placebo (to resemble CPA 50 mg tablets) taken daily from days 5–14 inclusive.

TABLE 1. Clinical details of patients taking part in the trial

| Group | Therapy | No. of patients entering trial (no. completing) | Age in years mean and (range) | Weight kg mean and (range) | Baseline SER ($\mu$g/cm$^2$/min) | Mean baseline acne grades | |
|---|---|---|---|---|---|---|---|
| | | | | | | Total | Facial |
| M | Minovlar and placebo | 28<br>(24) | 24·2<br>(18–36) | 58·7<br>(58·3–59·2) | 1·63 | 2·68 | 1·55 |
| D | Diane and placebo | 32<br>(26) | 24·2<br>(18–34) | 58·6<br>(45·8–74·1) | 1·56 | 2·73 | 1·54 |
| C | High dose cyproterone acetate | 30<br>(26) | 22·8<br>(16–30) | 55·9<br>(43·5–80·0) | 1·63 | 2·60 | 1·57 |

*Group D (Diane).* Cyproterone acetate 2 mg and ethinyl oestradiol 50 $\mu$g: a combined preparation taken daily from days 5–25 of the cycle inclusive; plus: placebo as in Group M.

*Group C (high dose CPA).* Cyproterone acetate 50 mg taken from days 5–14 of the cycle inclusive; ethinyl oestradiol 50 $\mu$g taken from days 5–25 of the cycle inclusive. Group C followed the reversed sequential regime of Hammerstein *et al.* (1975).

The following measurements were recorded for each patient at every visit unless otherwise stated:

*Severity of acne* on the face, back and chest was graded according to the method of Burke and Cunliffe (1984) by at least two observers. The separate grades were added up to give a total result. In addition, numbers of macules, papules, pustules, nodules and cysts on the face were counted, if possible by the same person at each visit, and blood pressure was recorded. Weight was recorded at initial and final visits.

*Sebum excretion rate* was measured by the method based on Pochi and Strauss (1961) with the modifications of Cunliffe and Shuster (1969) and Cunliffe, Williams and Tan (1975).

*Subjective changes* in acne severity were assessed using a 20 cm visual analogue scale. The 10 cm midpoint was taken as the baseline at the first visit, and scores above this value were used to indicate an improvement and scores below this value a worsening of the acne.

*Photographic assessment* was performed at all visits. These were ranked according to severity and graded by at least two observers using randomly-allocated photographs.

*Bacterial sampling* for anaerobes (*P. acnes*) and aerobes (staphylococci and micrococcaceae) from an area of the face of each subject was performed by the 'cup scrub' technique (Williamson & Kligman, 1965). Numbers of colony-forming units were determined by plating out serial decimal dilutions of the wash fluid onto tryptic soya agar recovery media. Bacterial numbers were expressed as $\text{Log}_{10}$ colony-forming units per cm$^2$. The counts were correlated with therapy, SER and clinical response.

### *Statistical methods*

The data for SER and bacterial counts were normally distributed. Comparisons between the treatment groups were carried out using one-way analysis of variance and *t*-tests of the differences between means. Comparisons within groups, at different times, were carried out using paired *t*-tests. The product-moment correlation coefficient and linear regression were used to investigate possible predictive qualities.

The distribution of the remaining data varied between a small degree of non-normality, as in the basic characteristics of age, weight, etc., to extremely non-normal, usually with very pronounced positive skews, as in the acne lesion counts. These data were analysed, therefore, by distribution-free methods. Comparisons between treatment groups employed the Wilcoxon rank sum test, the Kruskal-Wallis one-way analysis of variance by ranks, and the Komolgorov-Smirnov two-sample test for ordinal data for grades and lesion counts; the chi-squared test and the extended median test were used for nominal data including the numbers of patients on various pre-treatment medications. Comparisons between times, within treatment groups, were carried out using the Wilcoxon matched-pairs signed-ranks test. All tests were appraised one-tailed.

## RESULTS

From the three treatment groups, Minovlar (M), Diane (D) and high dose CPA (C), 24, 26 and 26 patients respectively completed the trial and were therefore included in the analyses. There were no differences between the three groups at the start of the study with respect to their previous usage of antibiotics and the contraceptive pill, their ages and initial acne grading (Table 1).

Nearly all the patients had taken antibiotics for acne at some stage and 71% had been or were currently taking the contraceptive pill. The only significant difference found between the groups was in the body weight ($P < 0.05$; Kruskal-Wallace test) (Table 1).

### *Clinical findings*

There was an improvement in both total (Fig. 1a) and facial acne grades (Fig. 1b) in all three groups during the 6-month assessment period. Significant reductions in total and facial grades had occurred in Groups D and C by 2 months and in Group M by 4 months. At 6 months Groups D and C had both shown a significantly greater improvement than Group M (Wilcoxon rank sum test).

These clinical findings for the facial grade were supported by the photographic assessment. Group C had shown a significant improvement by 2 months and Group D by 4 months (Fig. 2) (Wilcoxon matched pairs signed ranks test). Group M showed a trend towards improvement but this did not reach signficance at any time. By 6 months both Groups D and C had improved more than Group M ($P < 0.05$ and $P < 0.01$ respectively). The difference in the baseline grades between the clinical and photographic assessments can be attributed to the effect on the grades of lesions that were apparent only on palpation.

Individual lesion counts showed a reduction in numbers of papules (Table 2), with a statistically significant fall being recorded in Groups C and D by 4 months ($P < 0.001$ and $P < 0.005$ respectively; Wilcoxon matched pairs signed ranks test). The improvement in Group M was much less marked ($P < 0.1$). Macule counts also decreased in Groups C and D, but this did not reach statistical significance because of the wide ranges of counts (Table 3). With respect to both macules and papules, Group C had improved significantly more than the other two groups by 4 months. Although the number of patients with large numbers of pustules was small, there was a statistically significant improvement in Groups C and D by 2 months ($P < 0.01$),

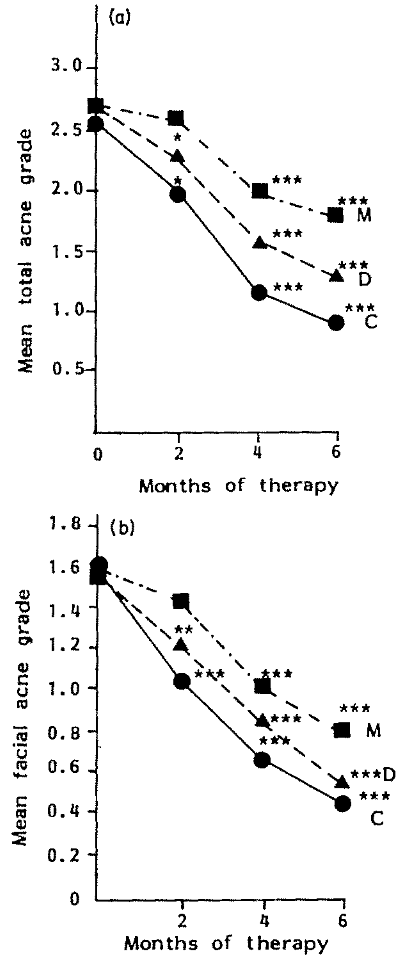

FIGURE 1(a). Mean total acne grades before and during therapy with high dose CPA (C), Diane (D), or Minovlar (M). C vs M $P<0\cdot01$; D vs M $P<0\cdot05$; C vs D n.s. * $P<0\cdot05$; ** $P<0\cdot01$; *** $P<0\cdot001$. (b) Mean facial acne grade before and during therapy with high dose CPA (C), Diane (D) or Minovlar (M). C vs M $P<0\cdot005$; D vs M $P<0\cdot05$; C vs. D n.s. ** $P<0\cdot01$.***$P<0\cdot001$.

which was not found in Group M until 6 months ($P<0\cdot005$; Wilcoxon matched pairs signed ranks test). The amount of data available on nodule and cyst counts was too small to analyse.

*Sebum excretion rate*

There was a marked reduction in the raised rate of sebum excretion in both Groups C and D by 2 months with only small further reductions thereafter (Fig. 3). Although the difference between Groups C and D was not significant, again the trend was for a greater reduction in Group C. In Group M there had been no significant reduction in SER by 6 months (paired $t$-test). No significant correlation was found in individual patients between the reduction in SER and subsequent clinical improvement (Spearman's rank correlation coefficient).

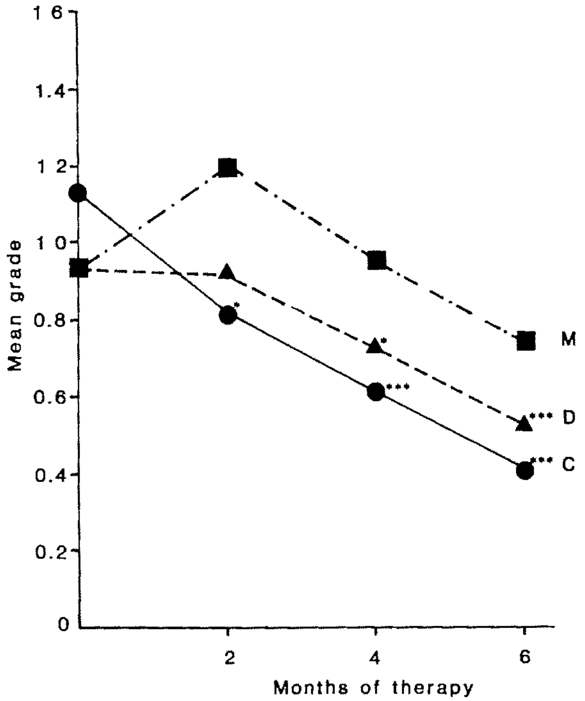

FIGURE 2. Mean facial acne grading assessed photographically before and during therapy with high dose CPA (C), Diane (D), or Minovlar (M). $*P > 0{\cdot}05$; $***\ P > 0{\cdot}001$.

TABLE 2. Total papule counts in the three treatment groups (median and interquartile range)

| | Group | | | |
|---|---|---|---|---|
| | C | D | M | Intergroup comparisons† |
| Baseline | 26·5 (13·75–48·5) | 33·5 (14·75–58·5) | 29·5 (16·0–45·0) | NS |
| 2 months | 22·5 (10·0–37·25) | 27·0 (16·0–52·75) | 26·5 (18·5–47·0) | NS |
| 4 months | 9·0 (7·0–12·25)*** | 13·5 (8·75–34·75)** | 24·0 (8·5–36·75) | D > C; $P < 0{\cdot}05$<br>M > C; $P < 0{\cdot}01$ |
| 6 months | 5·5 (3·0–15·5)*** | 15·0 (8·75–27·0)** | 19·5 (13·0–25·75) | D > C; $P < 0{\cdot}01$<br>M > C; $P < 0{\cdot}01$ |

$**P < 0{\cdot}005$; $***P < 0{\cdot}001$; Wilcoxon matched pairs signed ranks test.

† See details of statistical tests on p. 708.

TABLE 3 Total macule counts in the three treatment groups (median and interquartile range)

| | Group | | | |
|---|---|---|---|---|
| | C | D | M | Intergroup comparisons† |
| Baseline | 42·5 (20·75–84·75) | 63·0 (23·0–94·0) | 40·0 (29·0–61·5) | NS |
| 2 months | 57·5 (35·0–70·0) | 74·5 (38·5–105·0) | 56·0 (27·25–90·5) | NS |
| 4 months | 39·0 (20·5–66·5) | 51·0 (27·25–85·25) | 48·0 (21·75–81·5) | D > C $P < 0{\cdot}05$ |
| 6 months | 21·0 (7·75–53·0) | 39·0 (22·25–86·25) | 50·5 (33·5–95·25) | D > C $P < 0{\cdot}05$<br>M > C $P < 0{\cdot}05$ |

† See details of statistical tests on p. 708.

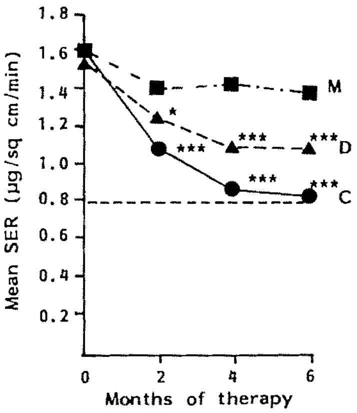

FIGURE 3. Mean SER before and during therapy with high dose CPA (C), Diane (D) or Minovlar (M). (– – – –) upper limit of normal range for females aged 18–22. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}001$.

### *Visual analogue score*

Subjective improvement was noted in all three groups. Group C achieved a better response than either Group D ($P < 0{\cdot}025$) or M ($P < 0{\cdot}05$) at 6 months (extended median test). Groups D and M were comparable.

### *Bacteriology*

A significant reduction in anaerobic and aerobic bacterial counts was noted in Group C at 4 months (Fig. 4). No significant changes were seen in Group M or D (paired *t*-test). No correlation was found between anaerobic and aerobic bacterial counts and total acne grade either at the baseline examination or in response to therapy (Spearman's rank correlation coefficient). There was a significant correlation between reduction in $\mathrm{Log}_{10}$ SER and a decrease in the anaerobic bacterial count in Group C ($P < 0{\cdot}05$) (Spearman's rank correlation coefficient).

### *Side-effects and patients failing to complete the study*

Eleven patients failed to complete the trial (Table 4). In Group M, four patients left the trial.

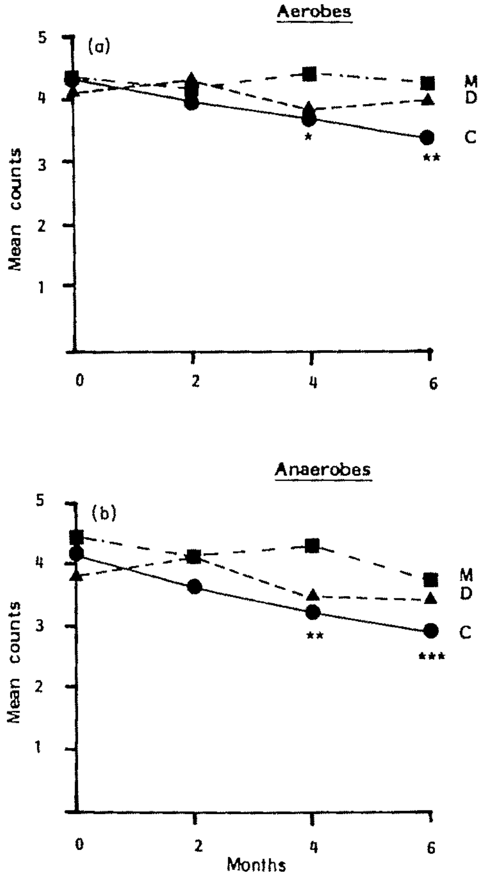

FIGURE 4. Aerobic and anaerobic bacteria from the face before and during therapy with high dose CPA (C), Diane (D) or Minovlar (M). Mean $\text{Log}_{10}$ colony-forming units per cm$^2$. (a) $*P < 0{\cdot}02$; $**P < 0{\cdot}005$ (b) $**\ P < 0{\cdot}01$; $***P < 0{\cdot}001$

One patient went abroad and three withdrew because of failure of therapy, two within 2 months of starting the trial.

In Group D, six patients left the trial. Three patients went abroad; in one patient the acne deteriorated within 2 months, one developed depression accompanied by diarrhoea and abdominal pain and was later diagnosed as having SLE, and the last patient complained of dizziness and found it difficult to attend the clinic.

In Group C, one patient left the trial because of a worsening of her acne. In addition, three patients in this group were not included in the statistical analysis because of lack of data from intermediate visits.

In none of the treatment groups was there any significant change in the patients' weight (paired *t*-tests). In 37 patients an increase in weight was recorded but in 30 there was a reduction. No significant changes in blood pressure were noted in any group.

The numbers of patients leaving the trial did not differ significantly between the groups (chi-squared test of proportions). Side-effects were difficult to assess because of their subjective

TABLE 4. Side-effects reported at one, or more than one, visit during the trial

| | No. of Patients | | | | | |
|---|---|---|---|---|---|---|
| | Diane<br>$n=26$ | | CPA<br>$n=26$ | | Minovlar<br>$n=24$ | |
| Symptoms | > once | once | > once | once | > once | once |
| Tiredness/lassitude | 1 | | 3 | 1 | 4 | 1 |
| Loss of libido | | | 1 | 1 | | 1 |
| Headaches | 1 | 3 | 3 | 4 | 3 | 2 |
| Dizziness/giddiness | 1 | 5 | | 4 | 1 | 3 |
| Breast soreness | 1 | 5 | 2 | 4 | 2 | 3 |
| Urgency of micturition | 1 | | 1 | 3 | | 3 |
| Depression | 1 | 3 | 1 | 3 | 1 | 3 |
| Nausea/vomiting | 2 | 6 | 2 | 2 | 2 | 6 |
| Abnormal uterine bleeding | 1 | 2 | 1*** | 4 | | 3 |
| Aching legs | | | | 3 | | 1 |
| Constipation | 1 | 1 | | | | 3 |
| Abdominal pain | 1* | | | | | |
| Benign breast tumour | 1** | | | | | |
| Vaginal discharge | 1*** | | | | | |

* Withdrew from trial due to side-effects.

** Patient reported lump halfway through trial, time of origin unknown (*see* text).

*** Both patients underwent D & C. Both found to have cervical erosion, thought to be unrelated to therapy.

nature. However, no major differences were found between the treatment groups with respect to total numbers of patients reporting side-effects or the incidence of individual side effects. In this trial seven of 26 patients in Group D, eight of 26 in Group C and six of 24 in Group M gave no history of side-effects at any stage. There was a highly significant tendency for side-effects to be reported at the second visit with a considerable reduction in complaints at later visits ($P < 0{\cdot}001$).

One patient in Group C complained of irregular menstrual bleeding and one in Group D gave a history of vaginal discharge and bleeding. Both underwent D & C and were shown only to have cervical erosions. One patient in Group D reported a breast lump which was removed subsequently and which showed fibroadenomatosis.

## DISCUSSION

The results of this randomized double-blind trial showed clinical improvement in the severity of acne in all three treatment groups, when assessed objectively by clinical gradings, photographic assessment and lesion counts, or subjectively by visual analogue scores. Despite the fact that all patients received the same dose of oestrogen, a more rapid and complete response was seen in those groups who were given CPA. Although clinical assessments failed to show statistically significant differences between Groups C and D, there was a consistent trend towards a more favourable outcome in those treated with the higher dose of CPA with respect to all the parameters measured. A dose-related response has been reported by other investigators (Breckwoldt *et al.*, 1978; Aron-Brunetiere, Dutouquet & Ventura, 1984), although it was suggested that after 12 months' therapy there was little difference between Diane and high dose CPA (100 mg) (Breckwoldt *et al.*, 1978). In the present study only 50 mg CPA was used in the high dose regime although several patients with recalcitrant acne appeared to achieve better clinical results when given 100 mg CPA after the trial had been completed.

The marked reduction in SER in Groups C and D was expected and the rapidity of this response has been noted before (Ebling *et al.*, 1977). We could not correlate the reduction in SER with clinical improvement in individual patients nor did our results support the findings of other investigators that Minovlar significantly reduced SER (Pye *et al.*, 1977).

The finding that a significant reduction in the bacterial population of the face occurred only in Group C while there were significant improvements in facial acne grade in all groups and a significant reduction in SER in Groups D and C, suggests a dissociation between clinical improvement and a reduction in SER and the bacterial population, and implies that reduction in the surface bacterial population is not a primary event in the success of anti-androgen therapy.

Adverse effects were equally distributed within the three treatment groups and therefore cannot be attributed specifically to the anti-androgen medication. Most of these adverse effects were those commonly seen during treatment with the oral contraceptive pill. Our results agree with those of most other workers who have reported no increase in the rate of side-effects with the use of CPA/oestrogen combinations when compared with oral contraceptives with a similar oestrogen content (Hammerstein *et al.*, 1975; Ekoe *et al.*, 1980; Aydinlik & Lachnit-Fixson, 1977; Lachnit-Fixson, 1979). However, the high dose CPA regime has been associated occasionally with an increased incidence of lassitude and reduction in libido (Hammerstein *et al.*, 1975). Side-effects were more common within the first 3 months of therapy (Breckwoldt *et al.*, 1978; Kaiser *et al.*, 1976) with a considerable reduction in incidence thereafter. CPA is a powerful progestagen and when combined with oestrogen provides good control of the menstrual cycle and is an effective oral contraceptive (Aydinlik & Lachnit-Fixson, 1977; Breckwoldt *et al.*, 1978; Lachnit-Fixson, 1979; Kaiser *et al.*, 1976). Although it might have a feminizing effect on a male foetus, no problems as yet have been reported in pregnancies that have occurred while patients have been taking CPA either in the high dose or Diane regimes (Schering Chemicals Ltd, personal communication).

## ACKNOWLEDGMENTS

We would like to thank Professor W.C.Noble, Department of Microbiology, Institute of Dermatology, St John's Hospital for Diseases of the Skin, for help with the bacteriological investigations and sebum excretion measurements, and Schering Chemicals Ltd for financial assistance.

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*British Journal of Dermatology* (1986) **114**, 717–728.

***Society Proceedings***

# Proceedings of the 234th Meeting of the Netherlands Society of Dermatology and Venereology, Rotterdam, 8 June 1985

CHAIRMAN: P.A.J.J.DE COCK
HOSTS: PROFESSOR TH. VAN JOOST
PROFESSOR E.STOLZ

Received 25 November 1985

## CASE REPORTS

### Pyoderma Gangrenosum

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*History.* Female, aged 6 years. Two year history of painless lesions scattered over the body, that healed spontaneously with scarring. She complained of recurrent abdominal pain and diarrhoea.

*Examination.* Erythematous papules and nodules with central necrosis and red undermined edges were seen on the right leg and the fingers (Fig. 1).

*Laboratory tests.* Blood cell counts, liver enzymes, immunoglobulins, protein analysis and vitamin levels were all within normal limits. Rheumatoid factors, ANF, circulating auto-antibodies and Crohn-serology were negative; urine and faeces analyses were normal. Slightly raised streptococcal antibodies were detected. Her tissue type was HLA $A_3$, $A_{24}$, $B_7$, $BW_{55}$, $CW_3$. The Mantoux test was negative and abdominal ultrasound was normal.

*Immunological studies.* Leukocyte-function tests, including chemotaxis, migration, oxygen consumption and phagocytosis, were all normal.

*Histology.* (i) Ulcerated lesion. The fragments of epidermis present showed ortho- and parakeratosis. Centrally extensive necrosis was seen in the dermis surrounded by an inflammatory infiltrate consisting of polymorphonuclear leukocytes. Many eosinophils were present and the vessels showed endothelial swelling. (ii) Taken from edge of fresh lesion on leg. The epidermis showed parakeratosis and acanthosis. In all dermal layers a perivascular lymphocytic inflammatory infiltrate was seen. There was marked endothelial swelling of the vessels.

*Direct immunofluorescence.* Biopsy 2: IgM and complement deposits were found along the basement membrane. Perivascular deposits of complement and fibrin were also detected.

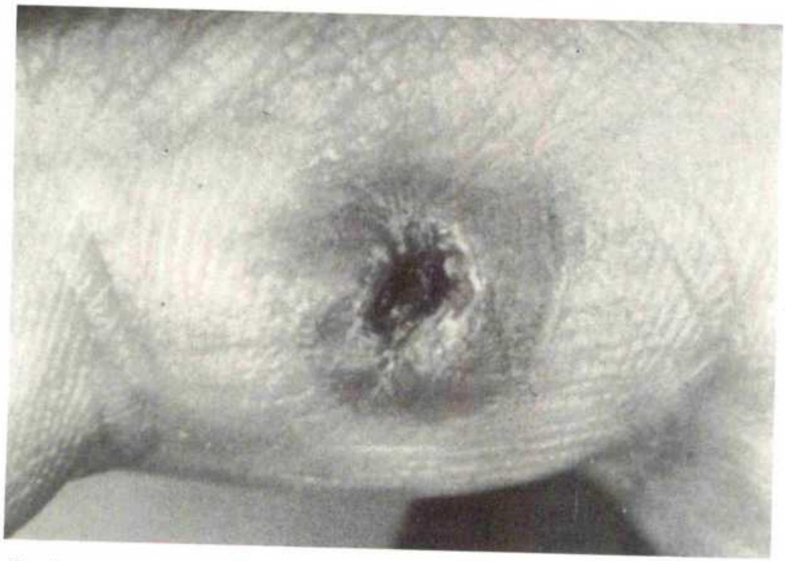

FIGURE 1. Erythematous papule with central ulceration and undermined edges: pyoderma gangrenosum.

*Discussion.* Pyoderma gangrenosum is an ulcerating skin disorder of unknown aetiology which can occur at any age, although reports of this disease in childhood are rare. Associations with systemic diseases have been described, including polyarthritis, Crohn's disease, ulcerative colitis, monoclonal gammopathy, chronic active hepatitis, leukaemia, polycythemia vera, lymphoma, systemic lupus erythematosus, diabetes mellitus and Wegener's granulomatosis. In 20–50% no association is found. In this case no signs of a systemic disease have yet been demonstrated.

## 'Moth-eaten' alopecia in a case of secondary syphilis

A.H. VAN DER WILLIGEN

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*History.* Male, aged 43 years. In 1983 the patient presented with complaints about progressive, diffuse loss of scalp hair and bald patches on the upper lip. Six weeks earlier, the patient had had a small ulcer on the penis which had disappeared spontaneously. He did not use any medication.

*Examination.* On the scalp the pattern of calvities frontalis et cranialis was present with, in addition, a typical 'moth-eaten' alopecia (Fig. 2). The eyebrows and the upper lip showed round hairless patches. The body hair did not show any abnormalities. A scar was seen at the base of the penis.

*Laboratory tests before treatment.* Syphilis serology: RPR positive 4+, TPHA positive, FTA-abs. positive 3+, VDRL positive 1:32. Hair root status: a decreased number of anagen hair roots and an increased number of catagen and telogen hair roots were found.

*Treatment.* The patient was treated with procaine-penicillin G 600,000 IU/day i.m. for 4 days, and on the fifth day with a combination of 600,000 IU benzathin-penicillin G, 300,000 IU procaine-penicillin G and 300,000 IU penicillin G i.m. This was continued for 2 weeks.

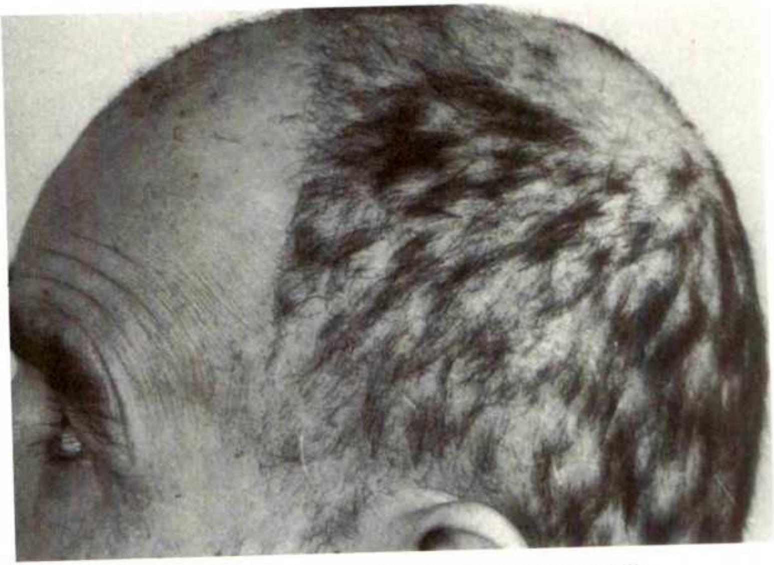

FIGURE 2. 'Moth-eaten' alopecia in secondary syphilis.

*Laboratory tests after treatment*. Four months later hair growth had recovered and syphilis serology was: TPHA positive, FTA-abs. positive 3+, VDRL positive 1:4.

*Discussion*. Two types of hair loss are recognized with secondary syphilis. There are the acute 'moth-eaten' alopecia and diffuse syphilitic alopecia. Acute 'moth-eaten' alopecia is clinically characterized by loss of tufts of hair resulting in numerous small bald patches. The clinical picture is compared with moth-eaten fur. Not only the scalp, but all hair-bearing areas of the body may be affected. Hair growth recovers spontaneously after a few weeks, even without therapy. A disturbed blood supply of the hair follicles by the inflammatory infiltrate is postulated as the cause. Three to five months after infection, diffuse loss of scalp hair may occur which is similar to the alopecia observed after febrile infectious diseases. On examination of the hair roots we saw a decrease in the number of anagen hair roots and an increase in the number of catagen, telogen and dysplastic/dystrophic hair roots. In a prospective study in our venereology department hair root assessment was undertaken in 10 primary syphilis and eight secondary syphilis patients. In both primary and secondary syphilis there is a statistically significant decrease in the number of catagen hair roots in the cranial and left temporal areas.

No difference between hair loss patterns in primary and secondary syphilis can be demonstrated. We may conclude that with a syphilitic infection the duration of the anagen phase is shortened and that consequently an accelerated transition takes place via the catagen phase to the telogen phase.

## Pemphigoid and adenocarcinoma of the pancreas

J. Muntendam, F.B. Blog and Th. van Joost

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*History*. Female, aged 59 years. In November 1984 the patient presented with a one-month history of pruritus.

*Examination*. Multiple, sharply defined, gyrated, erythematous patches with central clearing were seen

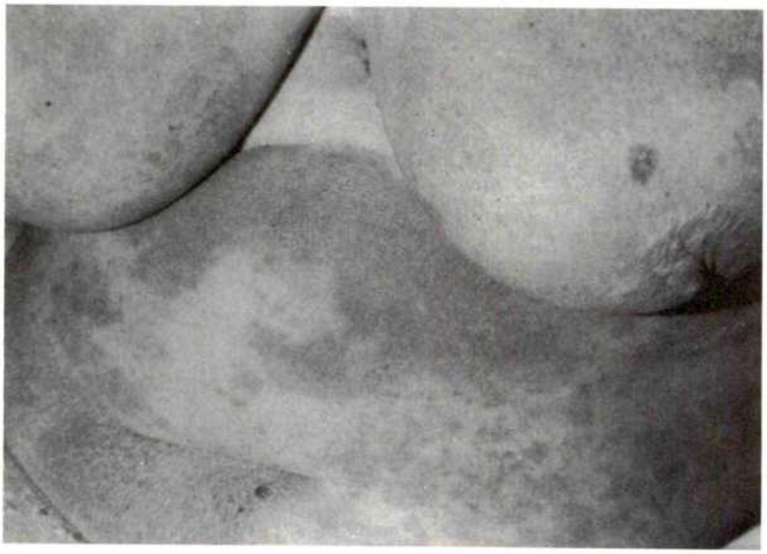

FIGURE 3. Erythematous irregular patches in parapemphigus associated with adenocarcinoma of the pancreas.

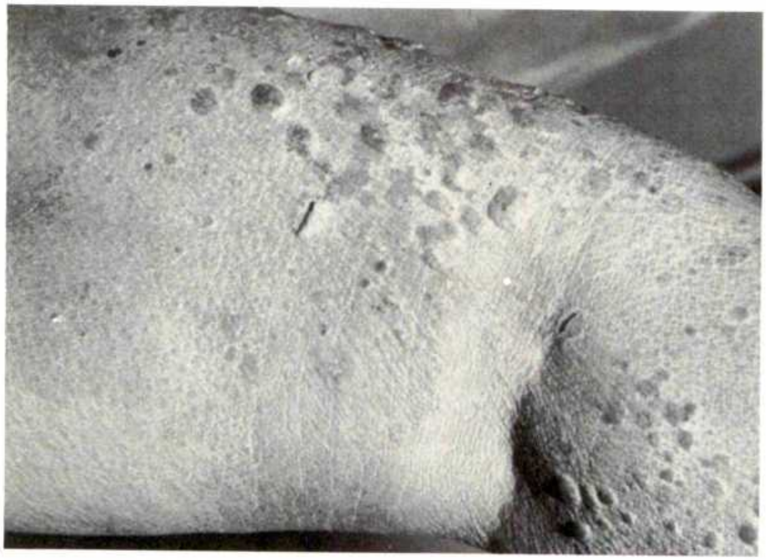

FIGURE 4. Multiple bullous lesions in parapemphigus associated with adenocarcinoma of the pancreas.

on the body, excluding the face (Fig. 3). In many areas there were multiple bullae (Fig. 4). The Nikolsky phenomenon was negative and the mucous membranes were normal.

*Laboratory tests.* Haematology: leukocytes $13{\cdot}9 \times 10^9$/l, with an eosinophilia of 15%. Renal and liver function tests were normal, except alk. phosphatase which was 70 U/l (normal, 18–45). Diff.alk. phos.: liver fraction slightly raised; gall bladder fraction slightly raised. LDH 468 U/l (normal, 150–350). CEA

360 μg/l (normal 0–5). The serum glucagon level was 46 ng/l (normal, 0–80). Indirect IF examination for circulating pemphigus or pemphigoid antibodies was negative. Bacterial, viral and fungal cultures of blister fluid were all negative.

Diabetes mellitus was diagnosed.

*Histology.* A sub-epidermal bulla filled with fibrin and the underlying inflammatory infiltrate contained many eosinophils. Around the blood vessels in all layers of the dermis a moderate to dense inflammatory infiltrate consisting of lymphocytes, plasma cells and segmental eosinophilic granulocytes was seen.

*Direct immunofluorescence.* Showed a moderate to strong linear fluorescence along the basement membrane of the epidermis with IgG, total complement and B1A.

These findings were considered consistent with bullous pemphigoid.

*Course of illness.* Because of the atypical clinical course with partial spontaneous regression of the skin lesions, a paraneoplastic dermatosis was suspected. A CT scan of the upper abdomen showed a space-occupying lesion in the pancreatic area and features suggestive of liver metastases. Light and electron microscopy of needle biopsy material from one of those areas showed a poorly differentiated adenocarcinoma, metastasis from a primary carcinoma of pancreas.

On the basis of the histology, a negative reaction with antiserum against glucagon and a normal serum glucagon level, metastases from glucagonoma could be excluded. The diabetes mellitus was treated with insulin. In view of the nature and the extent of the tumour, it was decided to refrain from further therapy in consultation with the internal medicine and oncology departments. The patient died early in January 1985.

*Discussion.* Opinions differ about the significance of the simultaneous occurrence of pemphigoid and malignancy. Some authors (Person & Rogers, 1977; Hodge *et al.*, 1981; Venencie, Rogers & Schroeter, 1984) suggest that among parapemphigus patients with a negative indirect immunofluorescence, a higher frequency of malignancy can be demonstrated than among patients with positive indirect immunofluorescence.

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## Crohn's disease of the vulva

J. VAN ULSEN, A. NOTOWICZ* AND A.P.C. VAN DER MAAS*

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*History.* Female, aged 29 years, from Surinam, and of Indian origin. In 1981 the patient developed small ulcers on the vulva accompanied by swelling and itching which persisted for a year. Under suspicion of lymphogranuloma venereum she had been treated with doxycyclin and subsequently with antituberculosis therapy for 9 months without success. The patient had no intestinal complaints.

*Examination.* The labia minora were swollen. In the anogenital area from the vulva to the perineum extensive ulceration was seen (Fig. 5). In the perineum and perianal area there were multiple polyps.

*Laboratory tests.* With specific histopathological staining procedures and bacteriological laboratory

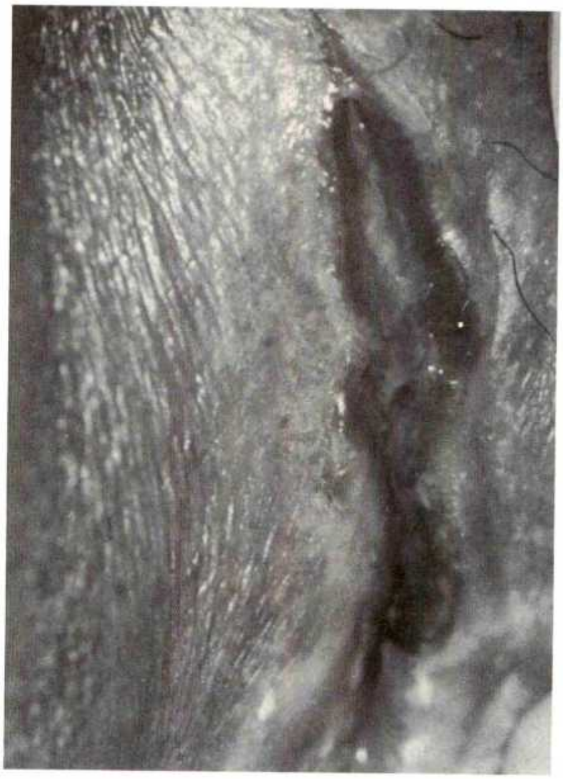

FIGURE 5. Deep ulceration in the groin: Crohn's Disease.

investigations most causes of granulomatous infections were excluded. Agglutination reactions with the patient's serum with anaerobic coccoid rods were strongly positive with ME44 and ME46. Proctoscopy in January 1982 showed no evidence of Crohn's disease.

*Histology*. Biopsy of an ulcer between left labium majus and inguinal fold showed a granuloma with epitheloid cells and giant cells of the Langerhans type.

*Treatment*. On suspicion of Crohn's disease the patient was treated with salazosulphapyridine and local corticosteroids with little effect. Three years later, in 1984, she was hospitalized elsewhere with intestinal complaints of frequent diarrhoea with blood and mucous, and abdominal pain. Proctoscopy showed severe colitis with red oedematous, easily bleeding mucous membranes with cobblestone aspect. Histological examination of intestinal biopsies showed a granulomatous process consistent with Crohn's disease.

*Discussion*. Vulvar localization as the first sign of Crohn's disease is very rare. In our patient the diagnosis was finally made after follow-up of more than 3 years.

## Cutaneous T-cell lymphoma, a variant of Hodgkin's Disease?

J.W.M.HABETS, F.B.BLOG AND TH.VAN JOOST

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*History*. Male, aged 42 years. Since 1981 he had had a generalized, slowly expanding ichthyosis. At the end of 1982 an ulcerating tumour developed on the left shoulder with, in addition, generalized erythemato-squamous lesions. On physical examination enlarged lymph nodes in the neck and the groin were found. After investigation the diagnosis of mycosis fungoides stage I with dermatopathic lymphadenopathy was established. The patient was treated with PUVA therapy. In October 1984, a relapse of the skin lesions occurred.

*Examination.* On trunk, limbs and face there were poorly defined erythemato-squamous lesions of varying size. The extensor aspects of the lower legs had substantial ichthyosiform scaling. There were enlarged lymph nodes in the left axilla.

*Laboratory tests.* DNA-cytophotometry of the tumour showed polyploidy (4 n 10%, 4 n 13%). Upon further examination no abnormalities suggestive of mycosis fungoides were found.

*Histology.* (i) A tumour biopsy in 1982 showed a lymphocytic infiltrate consisting of atypical lymphocytes. No clearly demonstrable Pautrier micro-abscesses were present. Electron microscopy showed atypical T cells with cerebriform nuclei. (ii) A biopsy of the erythemato-squamous eruption was consistent with poikiloderma. Electron microscopy revealed no atypical cells. (iii) The lymph node was consistent with dermatopathic lymphadenopathy. (iv) A biopsy of the erythemato-squamous eruption in 1984 was consistent with parapsoriasis, with no atypical cells. (v) A lymph node biopsied in 1984 showed a background of dermatopathic lymphadenopathy and numerous areas containing Reed-Sternberg-like cells. On immunohistochemical examination these cells were Ki-1 positive. There was no evidence of systemic spread.

*Treatment.* Was with twice-weekly PUVA.

*Discussion.* The simultaneous occurrence of mycosis fungoides and Hodgkin's disease has been described by Hawkins, Schinella and Schwartz (1983). A similar case was described by van der Putte *et al.* (1982). The histology of the skin of this patient was consistent with mycosis fungoides. He developed later an immunoblastic lymphoma, and in a lymph node a Hodgkin's-like histology was seen. We conclude that our patient has a primary cutaneous T-cell lymphoma with a Hodgkin's-like histology of the lymph node. The acquired ichthyosis is interpreted as a paraneoplastic phenomenon.

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## Oral and vulvar aphthous ulcers

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*History.* Two female patients, aged 31 and 29 years, suffered for many years from oral aphthae. During and/or shortly after pregnancy, genital ulcers also developed. Patient 1 came from Thailand, Patient 2 was of Dutch origin.

*Examination.* Both patients had small superficial ulcers in the mouth and on the tongue (Fig. 6), upper lip, palate and buccal mucous membranes. The first patient also had a 3 cm diameter ulcer on the right labium minus (Fig. 7); the second patient had a 1 cm diameter ulcer on the left labium majus.

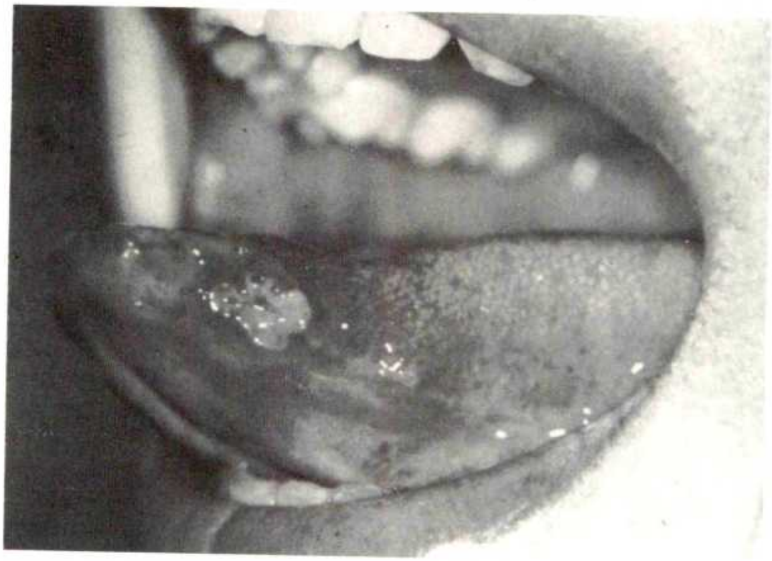

FIGURE 6. Ulcer on the tongue.

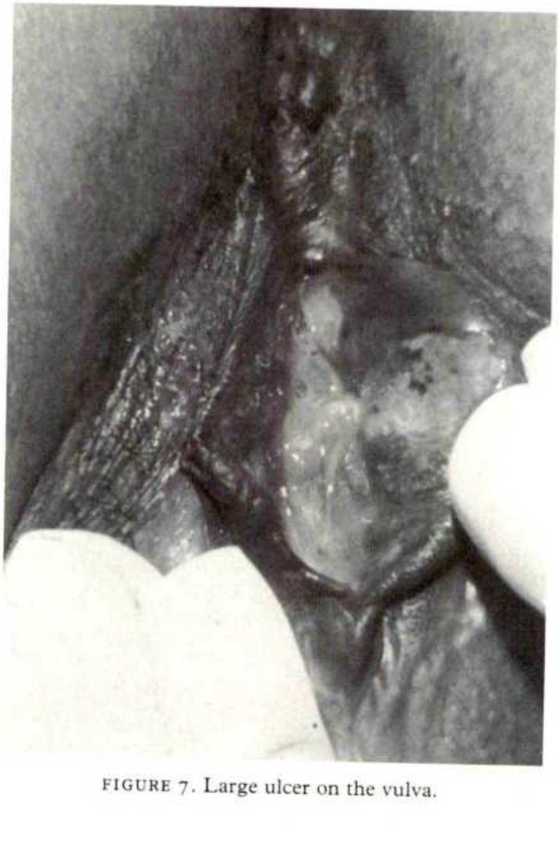

FIGURE 7. Large ulcer on the vulva.

*Laboratory tests.* In Patient 1 eosinophilia, slight vitamin A and E deficiency and a faecal hookworm infestation were found. In Patient 2 *Candida albicans* and *Torulopsis glabrata* were found in mouth and vagina respectively. Further laboratory examinations were negative.

*Histological examination.* Biopsies of ulcers in the mouth and on the genital sites showed an ulcerating, chronic, non-specific inflammation.

*Treatment.* The patients received specific treatment for their parasitic and fungal infections. Patient 1 also received vitamin A and E supplements. A comprehensive range of local and systemic therapies were given for the ulceration consisting of local antiphlogistic, antimycotic and antiseptic preparations and antibiotics with varying and temporary success. Patient 1 received prednisone 7·5–10 mg per day orally as a maintenance dose after delivery. In Patient 2, who requested treatment during pregnancy, a caesarean section was performed. The genital ulcers healed thereafter but the oral lesions persisted. The patient returned one year later with a recurrence of the genital ulcers, which healed with oral prednisone therapy.

*Discussion.* Oral aphthous ulcers are a common disorder. In some cases genital ulcers also develop, making the differential diagnosis with Behçet's disease difficult.

## SUMMARY OF PAPERS

### Evidence for an extra-cellular layer around freshly harvested *Treponema pallidum* (Nichols)

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The development of IgG anti-treponema antibodies as delineated in the FTA-ABS test in various stages during the classical course of human syphilis was presented. In all four IgG subclasses anti-treponema antibodies were found, present maximally during the secondary stage. It was shown that, in a FTA-ABS procedure employing treponemata spontaneously fixed to cultured cells or fibronectin layers, the treponemata failed to show fluorescence upon incubation with primary seropositive or secondary stage patient sera and conjugate. A minority of the treponemata showed a weak positive fluorescence following the incubation with a DEAE-sephadex isolated IgG preparation from a pool of secondary syphilis patients' sera, but the majority of the treponemata still failed to show a positive fluorescence in this situation. In

TABLE 1. Percentage of fluorescent freshly isolated or *in vitro* aged treponemata adhering to cultured human fibroblasts or human fibronectin after incubation with serum or IgG

| | Fibroblasts | | Fibronectin | |
|---|---|---|---|---|
| | Freshly isolated | *In vitro* aged | Freshly isolated | *In vitro* aged |
| L II serum | 0 | 0 | 0 | 0 |
| IgG (L) | 6–37* | 47–90* | 0–10* | 5–30* |
| Control serum | 0 | 0 | 0 | 0 |

*The numbers indicate the variation obtained in six separate experiments with different isolates of treponemata.

experiments performed after 24 h *in vitro* ageing of the treponemata, a somewhat larger percentage of the treponemata showed a positive fluorescence after incubation with IgG and conjugate; upon incubation with the syphilitic sera still no positive fluorescence was detected (Table 1). These results indicate that antibodies directed against surface components of treponemata do not combine with their epitopes on treponemata, fixed to the underlayer in the way described above. This suggests that these epitopes are covered with an extra-cellular layer, covering the treponemata. Since freshly harvested, 100% viable, treponemata were used, it seems likely that the results also apply to the *in vivo* situation. The increase in percentage of fluorescent treponemata suggests that the protective layer is lost during *in vitro* ageing, a phenomenon also known from other serological reactions, employing live treponemata, such as *Treponema pallidum* agglutination and *Treponema pallidum* immobilization reactions. These results show that *in vitro* results of serological reactions might not be directly applicable to the *in vivo* situation. The differences in results between tests performed with sera and IgG fractions suggest the presence of inhibitors of fluorescence in syphilitic sera.

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## Differentiation antigens in normal and psoriatic skin

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The distribution of receptors of keratinocytes in psoriatic epidermis and normal epidermis was studied using three new monoclonal antibodies, 253-B7, 224-C6 and 12-G7, and immunoperoxidase (IP) techniques. Monoclonal antibodies (Mcab) 253-B7 and 224-C6 were obtained by immunization of mice with squamous cell carcinoma and Mcab 12-G7 was obtained by immunization with mesothelioma cells. These were tested on frozen sections of skin biopsies from 10 healthy individuals (normal epidermis) and 10 patients with psoriasis (involved lesional and uninvolved epidermis).

Mcab 253-B7 and 12-G7 appeared to be markers specific for the epidermal basal cell layer (membrane and/or cytoplasm). Mcab 224-C6 stained only the cytoplasm of all 'suprabasal' cell layers. By use of Mcab 253-B7 an identical staining was seen in normal epidermis and uninvolved psoriatic epidermis. This was also the case using Mcab 224-C6. However, using Mcab 253-B7 and the IP technique, different cell layers in the lower epidermal regions were stained. This pattern was reproducible in several sections of psoriatic lesions and the findings might therefore suggest an increase of the germinal cell pool in lesional psoriatic epidermis.

The three Mcab were also tested on normal kerotinocyte cultures (Rheinwald & Green, 1975), and cell lines of an epidermoid carcinoma (A-431). All three Mcab stained cultured normal keratinocytes and A-431 cell lines. Mcab 12-G7 reacted with the HSC-1 cell line, but Mcab 253-B7 did not (Mcab A-431 was not tested on HSC-1). This finding suggests that Mcab 253-B7 and 12-G7 are reacting with heterogeneous antigens in germinal keratinocytes.

With immunoprecipitation techniques by use of $^3$H leucine labelled cultured keratinocytes and Mcab 253–B7, six bands were seen between 40–60 kd.

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## A comparison of two culture methods for the diagnosis of chlamydial infections

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A comparison was made of two culture methods for detecting *Chlamydia* in cervical and male urethral specimens. The first involved inoculating patient material in two separate vials. After 3 days, one monolayer was stained with Giemsa and the other one was disrupted for subpassage on a fresh monolayer. The second method was a modification of the rapid, simple microtitre technique, as has been described previously by Stamm (1983). Patient material was inoculated on a monolayer, grown in a microtitre plate (2 wells/sample). After two days the plate was stained with commercially-available fluorescent monoclonal antibodies (Microtrak®). Out of 932 samples, 66 were positive in the vials and 109 in the microtitre plates. Forty-nine samples were positive in both methods. From our data it appears that the sensitivity of two well-known culture methods may differ significantly, although both methods are considered to be acceptable. The microtitre technique with immunofluorescent staining should be used as reference method for the diagnosis of chlamydial infections.

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## Demonstration of treponemata and characterization of the inflammatory infiltrate in skin biopsies from patients with early syphilis

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Biopsies from 23 patients with early syphilis, proven by a positive dark-field preparation or serology, were tested for the presence and localization of treponemata. The composition of the inflammatory infiltrate in these biopsies was analysed on slides from parts of the biopsies, snap frozen in liquid nitrogen, using commercially available monoclonal antibodies against immunoglobulins (Dako) and T cells and T-cell subpopulations (Becton & Dickinson). In addition, the slides were also investigated for the presence of immunoglobulins and complement-factors on the treponema organisms. For staining treponemata in the biopsies, several histochemical (HC) and immunohistochemical (IC) methods were used and compared with each other. For IC methods, antisera against the Nicoll strain of treponema, raised in rabbits, were used. From the tested histochemical methods (Warthin, Dieterle, Levaditi, Steiner) that of Steiner was the most rapid, sensitive and reproducible.

In 13 out of 15 patients with primary syphilis many treponemata could be detected in the biopsies, mainly in the superficial dermis and epidermis, using HC as well as IC techniques. In the biopsies from eight patients with secondary syphilis, treponemata could be detected in seven in greatly varying numbers using the IC technique and in five using the HC technique.

In none of the biopsies could immunoglobulins or complement-components be demonstrated adjacent to the treponema organisms. In both primary and secondary disease the main components of the infiltrate were T cells. In eight out of 15 biopsies of primary syphilis patients T-helper/inducer (Leu3 positive) cells were more numerous than T-cytotoxic/suppressor cells (Leu2), while in three, Leu3 positive cells were about equal to Leu2 positive cells and in four, Leu3 positive cell numbers were less than Leu2 positive cells. In contrast, in biopsies of patients with secondary syphilis in three out of eight, Leu2 positive cells were most frequent, while in another three, Leu2 positive cell numbers were equal to Leu3 positive cells, and in two Leu2, positive cells were less common than Leu3 positive cells.

## The use of solid phase enzyme immunoassay (EIA) for the detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and herpes simplex virus

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The investigation was made with research prototypes of the solid phase enzyme immunoassay (EIA) (Abbott). These tests take approximately 4 h. The EIA for the detection of gonorrhoea was compared in 609 screenings of prostitutes (prevalence of gonorrhoea 8 7%) with the cultivation method on Thayer Martin (modified) and chocolate agar. For cervix samples the sensitivity was found to be 88%, the specificity 99% and the predictive value of a negative test 99%. The EIA to detect *Chlamydia trachomatis* antigen was evaluated in 403 men with other venereal disease (prevalence 19%) and 135 women (prevalence 17%). The EIA was compared with the culture on DEAE dextran-treated Hela 229 in vials, with subpassage, Giemsa stained. Of the total group of men the sensitivity was 70%, the specificity 98·5%. For the women this was 70% and 92%, respectively.

In men with the same diagnosis the prevalence differed enormously with age; for instance, in patients older than 30 years non-specific urethritis (NSU) had a prevalence of 17% and post-gonorrhoeic urethritis (PGU) a prevalence of 10%; in patients younger than 30 years the prevalences were 38% and 48% respectively. Of men younger than 20 years with PGU, 80% were positive for *Chlamydia trachomatis*. The test scored 100% sensitivity for cultures with more than 20 inclusions per slide, and 54% for cultures with less than 20 inclusions per slide. The EIA for the detection of herpes simplex virus (HSV) was evaluated in 154 patients with genital lesions (ulcers, crusts, vesicles, erythema) (prevalence 44%) against the cultivation method on two-cell cultures (Vero and HFL). One sample (a) was divided and used for EIA and culture; a subsequent sample (b) was only used for the culture.

In comparison of EIA with the results of cultures of both samples (a and b), the sensitivity was 80% and the specificity 100%. In comparison of EIA with the result of the culture of sample (a) only, then the sensitivity was 84% and the specificity 96%.

These three EIA tests offer various perspectives. The EIA to detect *Neisseria gonorrhoeae* antigen is very suitable for screening large groups of promiscuous women. The EIA for *Chlamydia trachomatis* antigen is not yet ideal because of low sensitivity. The EIA for the detection of HSV antigen requires further evaluation. The test is probably more sensitive in the case of vesicles or in an early erosive stage. The sensitivity in such cases is probably high enough to make the test of value.

## Sensitization to epichlorohydrin and epoxyresin

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To be published elsewhere.

## Bullous immunodermatoses and nephropathy

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## *Case Reports*

# Exacerbation of pemphigus by rifampicin

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### SUMMARY

Rifampicin therapy caused exacerbation of skin lesions, rising serum pemphigus antibody titres, and decreased serum betamethasone levels in a 59-year-old man with pemphigus vulgaris. Exacerbation of pemphigus was confined to the period of rifampicin therapy and seems to be best explained by the effect of rifampicin on the metabolism of betamethasone. Physicians prescribing rifampicin for the treatment of tuberculosis should be aware of its potential to produce such an important adverse reaction.

Rifampicin and glucocorticoids are sometimes prescribed in combination for the treatment of tuberculosis co-existent with steroid-responsive diseases. Clinically important decreases in the glucocorticoid effects of cortisol and prednisolone during rifampicin therapy have been reported during the treatment of adrenal insufficiency (Edwards *et al.*, 1974; Elansary & Earis, 1983), nephrotic syndrome (Hendrickse *et al.*, 1979), and pericarditis (van Marle, Woods & Beeley, 1979). Recently, we noted progressive exacerbation of pemphigus vulgaris during treatment with rifampicin.

### CASE REPORT

In July 1978, a 53-year-old man presented with a 2-month history of a blistering eruption over the trunk and limbs, and eroded areas in his mouth. The bullae, both ruptured and intact, were surrounded by areas of erythema. He was admitted to hospital. Biopsy of an early lesion showed suprabasal bulla formation and acantholysis. Direct immunofluorescence showed epidermal intercellular deposits of IgG, IgM, IgA and C3. Indirect immunofluorescence of the patient's serum, using normal human skin sections as the substrate, demonstrated circulating anti-epidermal intercellular antibodies (pemphigus antibodies) at a titre of 1:40. The biopsy and immunofluorescence results were consistent with a diagnosis of pemphigus vulgaris. The patient was treated with 5 mg of betamethasone daily and his skin eruption responded rapidly. The dosage was reduced to 0·5 mg daily on discharge in October 1978. Over the next 6 years his

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betamethasone dosage was maintained at 0·5–1·25 mg daily, with only occasional blister formation.

In October 1984, the patient was found to be suffering from pulmonary tuberculosis and a daily regimen of 450 mg of rifampicin, 400 mg of isoniazid and 750 mg of streptomycin was instituted. At this time he was also receiving 1 mg of betamethasone daily. On the 8th day of the treatment for tuberculosis, he noticed new blisters and eroded areas in his mouth and on his limbs. Thereafter, control of the pemphigus was lost and the dose of the steroid had to be increased. Discontinuation of streptomycin had no effect and on the 60th day, in spite of a dosage of 5 mg of betamethasone daily, further blister formation continued. It was then suggested that the exacerbation of pemphigus might have been caused by the effect of rifampicin in increasing the degradation of betamethasone. On this basis, rifampicin therapy was stopped and ethambutol (1000 mg/day) substituted. No other changes in therapy were made.

His eruptions improved rapidly, and 2 weeks after stopping rifampicin the skin lesions had virtually disappeared. Betamethasone dosage was gradually reduced to a maintenance dose of 1 mg daily by the end of April 1985. Only isoniazid and ethambutol have been continued for the treatment of his pulmonary tuberculosis. The results of a sputum culture for *Mycobacterium tuberculosis* were negative by this time and a chest X-ray disclosed pulmonary tuberculosis of minimal activity.

### *Serum betamethasone levels*

Serum concentrations of betamethasone were measured using a specific radioimmunoassay as described previously (Kano, Mizuchi & Miyachi, 1978). Blood samples were drawn at around 1400 h and sera were stored at −80°C until used. All the samples were assayed at the same time.

As shown in Table 1, the concentration of betamethasone in serum sample 3, obtained on the 13th day of rifampicin therapy, was considerably lower than those in serum samples 1 and 2,

TABLE 1. Pemphigus antibody titres, serum betamethasone levels, and daily dose of betamethasone, in relation to rifampicin therapy

| Serum sample | | Pemphigus antibody titre | Serum betamethasone level (ng/dl) | Dose of oral betamethasone (mg/day) |
|---|---|---|---|---|
| No. | Date taken | | | |
| Before rifampicin | | | | |
| 1 | June 20 1984 | 80 | 240 | 1·25 |
| Rifampicin started Nov. 7 1984 | | | | |
| 2 | Nov. 10 1984 | 320 | 172 | 1 |
| 3 | Nov. 20 1984 | 160 | 98 | 1 |
| 4 | Dec. 15 1984 | 320 | 272 | 3 |
| 5 | Dec. 25 1984 | 320 | 142 | 3 |
| Rifampicin withdrawn Jan. 8 1985 | | | | |
| 6 | Jan. 16 1985 | 640 | 1500 | 5 |
| 7 | Jan. 29 1985 | 160 | 1300 | 4 |
| 8 | Feb. 25 1985 | 160 | 660 | 2 5 |
| 9 | Mar. 11 1985 | 10 | 314 | 2 |

obtained before and on the 3rd day of rifampicin therapy respectively. This tendency was demonstrated even more clearly in serum samples 4 and 5 taken when the patient was receiving 3 mg of betamethasone per day. The levels of betamethasone in these samples were lower than that in sample 9, taken when the patient was receiving 2 mg of betamethasone daily.

## COMMENT

Rifampicin has been reported to cause pemphigus. Gange *et al.* (1976) reported the occurrence of pemphigus vulgaris together with liver function abnormalities about 9 months after rifampicin was started. Both abnormalities disappeared following rifampicin withdrawal. In a case of rifampicin-induced pemphigus foliaceus reported by Lee, Lim and Kang (1984), skin lesions developed 7 months after starting the drug. The mechanism by which rifampicin induced pemphigus in these patients remains unclear.

In our patient, short-term rifampicin therapy was associated with exacerbation of skin manifestations, rising serum pemphigus antibody titres and decreased betamethasone levels. These effects occurred within 2 weeks after beginning the treatment and reversed rapidly when rifampicin therapy was stopped. Although we were not able to determine directly the rate of breakdown of betamethasone given to the patient, these observations are consistent with the hypothesis that rifampicin acts to diminish the pharmacological effects of glucocorticoid therapy. Rifampicin induces a gradual proliferation of hepatic cell smooth endoplasmic reticulum, which is thought to be a locus of drug-metabolizing enzymes (Jezequel, Orlandi & Tenconi, 1971). The peak effect occurs after about 1–3 weeks of therapy (Ohnhaus & Park, 1979). Rifampicin increases the catabolism of drugs metabolized in the liver, thereby making the usual therapeutic doses of these drugs less effective (Edwards *et al.*, 1974; Nocke-Finck, Breuer & Reimers, 1973; Buffington *et al.*, 1976; Bergrem & Refvem, 1983).

In light of these observations, we conclude that an increased rate of degradation of betamethasone was responsible for the exacerbation of clinical manifestations in our patient during rifampicin therapy. To the best of our knowledge, our patient is the first case in which rifampicin is thought to have precipitated an acute exacerbation of pemphigus, and our data indicate that an increase in the maintenance dose of betamethasone by as much as five-fold was insufficient. From this experience, we suggest that alternative therapy, such as isoniazid in combination with ethambutol, should be employed when active tuberculosis is complicated by pemphigus.

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# Methotrexate toxicity precipitated by azapropazone

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## SUMMARY

We report an episode of bone marrow hypoplasia following the administration of azapropazone to a patient stabilized on regular methotrexate for psoriasis. A pharmacokinetic interaction is postulated.

Methotrexate is used in the management of severe psoriasis and in various malignancies. Azapropazone is a uricosuric, non-steroidal anti-inflammatory agent increasingly used in gout and other arthritic diseases (Dudley Hart, 1978). It has precipitated toxicity of anti-convulsants (Roberts *et al.*, 1982; Geaney *et al.*, 1982), totbutamide (Andreason *et al.*, 1981) and warfarin (Powell-Jackson, 1977).

## CASE REPORT

A 30-year-old woman was admitted with a 4-day history of oral and genital mucosal ulceration, sore throat and bloody diarrhoea. For 4 years she had taken oral methotrexate (25 mg weekly) for psoriasis unresponsive to other therapy. She had suffered two myocardial infarctions and had undergone coronary artery bypass grafting at 25 years of age. She was currently receiving dipyridamole 300 mg/day, aspirin 300 mg/day and clofibrate 1500 mg/day for hypercholesterolaemia. Two weeks previously she had had pains in the joints and had been given azapropazone in a dose of 2·4 g on the first day, 1·8 g on the second and then 1·2 g for 7 days. She had taken her methotrexate 8 days after the first dose of azapropazone.

She was pale and pyrexial. There were numerous superficial, painful oral and genital mucosal erosions and gross oral and vaginal candidiasis. The abdomen was diffusely tender and there were large plaques of psoriasis over the body. The scalp and nails were affected but there was no joint involvement. Haemoglobin was 8·2 g/dl, total white cell count 0·09 × $10^9$/l (all lymphocytes) and platelet count 54 × $10^9$/l. The bone marrow was grossly hypocellular with occasional clumps of erythroblasts and myeloblasts but no megakaryocytes. Marked eosinophilia was seen but no erythroid or granulocyte maturation. Plasma methotrexate concentration at this time was < 10 ng/ml.

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Management consisted of transfusion with red cells and platelets, analgesics, antibiotics and topical nystatin. Folinic acid (6 mg/m$^2$ 8-hourly intravenously) was given until the methotrexate concentration was known. Six days after admission the pyrexia settled, platelet count was normal and neutrophils were present in the blood. Three days later the neutrophil count was $1{\cdot}9 \times 10^9$/l. By 15 days the blood count was completely normal on no drug treatment. The diarrhoea and abdominal tenderness had settled by the third day. Subsequently, double contrast radiology revealed no ulceration of the small bowel. Liver biopsy 3 months previously had shown no evidence of hepatotoxicity and serum liver function tests remained normal.

## DISCUSSION

The typical clinical and haematological findings and rapid recovery indicate acute methotrexate toxicity. The absence of any reaction on the same dose and the close relationship to azapropazone therapy suggest a pharmacokinetic drug interaction.

Azapropazone is 95% bound to the warfarin binding site in plasma albumin (Fehske *et al.*, 1980). It can displace warfarin (McElnay & D'Arcy, 1977) and phenytoin (Geaney *et al.*, 1983) from this site and, presumably, other drugs as well. Methotrexate is bound to the same site but only to the extent of 50–70% (Bleyer, 1977). Such displacement has been invoked as a mechanism of interaction with salicylates (Taylor & Halprin, 1977) and sulphonamides (Liegler *et al.*, 1969) and in the precipitation of methotrexate toxicity by phenylbutazone (Adams & Hunter, 1976). Clofibrate is also extensively bound to plasma albumin and may have limited the available binding sites (Schrogie & Solomon, 1967). However, methotrexate is widely distributed so it would seem unlikely that sufficient could be displaced to increase substantially its concentration in the extracellular fluid.

Azapropazone inhibits the hepatic degradation of phenytoin (Geaney *et al.*, 1983) tolbutamide (Andreasen *et al.*, 1981) and antipyrine (Williamson *et al.*, 1984). After oral administration methotrexate is metabolized in the liver and gastro-intestinal tract to the extent of about 35% (Bleyer, 1977). Various metabolites are produced and a hydroxylated derivative has been identified. It is possible that azapropazone caused a decrease in clearance of methotrexate by inhibiting oxidative metabolism in the liver. This effect could have been enhanced by the concurrent clofibrate treatment which has also been implicated as an enzyme inhibitor (Skovsted *et al.*, 1974).

Both azapropazone and methotrexate are weak organic acids. The major route of elimination of both is by renal excretion involving active secretion into the nephron tubule (Bleyer, 1977; Jones, 1976). It is known that probenecid reduces the renal clearance of methotrexate (Aherne *et al.*, 1978) and the possibility of this type of interaction with other organic acids has been raised before (Liegler *et al.*, 1969).

The relationship between the kinetics of methotrexate and toxicity is complex. It appears that there is both a threshold drug concentration and a threshold duration of exposure to that concentration above which toxicity may occur (Bleyer, 1977). It is possible that in this patient a combination of pharmacokinetic interactions increased the marrow exposure to methotrexate to a level at which toxicity could occur.

Reports of serious drug interactions with azapropazone are increasing. Many patients with psoriasis are treated with methotrexate and so great care is needed in choosing an anti-inflammatory agent if there is associated arthropathy.

## ACKNOWLEDGMENT

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# Late metastases of cutaneous malignant melanoma

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## SUMMARY

Although late recurrence of cutaneous malignant melanoma (CMM) is a known clinical phenomenon, only 12 adequately documented cases exist in the literature. We describe two female patients with CMM who developed melanoma metastases 18 years after radical excision of their primary tumours and presumed cure. Increasing numbers of reports of cases of delayed metastases of CMM might have implications for management and follow up of melanoma patients.

Although in virtually all studies, prognostic factors important in predicting the outcome of stage I melanoma are related to a 5-year period, clinically a 10-year disease-free interval after excision of a primary (or metastatic) cutaneous malignant melanoma (CMM) is statistically and generally accepted as a presumed cure of the disease (Balch *et al.*, 1985; MacKie, 1983).

Late recurrence of CMM (after more than 10 years) is a rare but known phenomenon (Koh, Sober & Fitzpatrick, 1984). We describe two female patients who developed melanoma metastases 18 years after radical excision of their primary CMM.

## CASE REPORTS

### *Case 1*

In 1967, a 45-year-old woman presented with a pigmented lesion on the anterior side of her left lower leg. The clinical diagnosis was melanoma. The tumour was excised with a 3 cm margin and skin grafting, and histopathology revealed a Clark level III superficial spreading melanoma. Retrospective pathological reading of the original histopathological slides, and of newly performed sections from the original paraffin blocks, showed a tumour with a 2·2 mm Breslow thickness (Fig. 1). The site of the primary tumour and the left inguinal lymph nodes were prophylactically irradiated. No signs of distant metastases could be detected on X-ray examination.

The patient was followed up regularly in subsequent years and remained free from any signs of recurrence until 1985 when she noticed a deep, indolent and firm subcutaneous nodule on her

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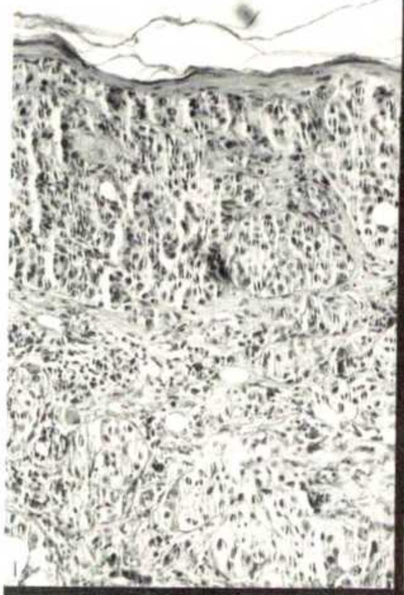

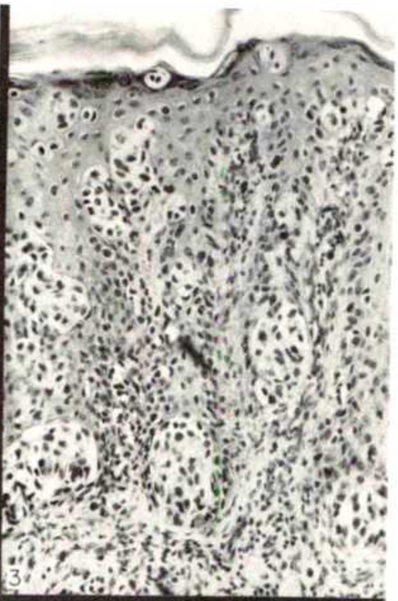

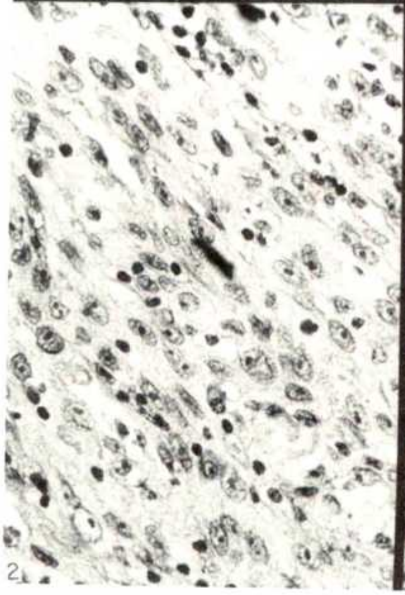

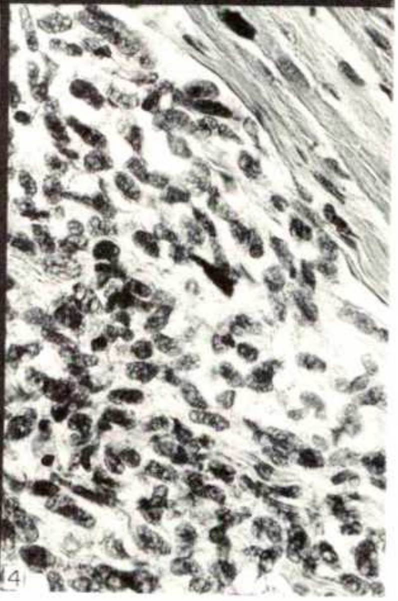

FIGURE 1. Case 1, 1967. Superficial spreading melanoma; Clark level III; Breslow thickness 2·2 mm. (H & E, original × 250).

FIGURE 2. Case 1, 1985. Cutaneous melanoma metastasis; aggregates of atypical amelanotic melanocytes in the dermis. (H & E, original × 400).

FIGURE 3. Case 2, 1966. Superficial spreading melanoma; Clark level IV; Breslow thickness 1·75 mm. (H & E, original × 250).

FIGURE 4. Case 2, 1984. Cutaneous melanoma metastasis; nests and aggregates of tumour cells in the dermis. (H & E, original × 400).

left calf. This was slightly proximal to the site of the original melanoma. The subcutaneous nodule was excised, and histopathology revealed a melanoma metastasis (Fig. 2). A careful dermatological investigation failed to reveal a second primary melanoma, and thorough screening, including internal, gynaecological, ophthalmological and neurological examinations, blood biochemistry, chest X-ray, abdominal ultrasonography, whole body scintigram, computer tomography and brain scan, did not reveal any additional melanoma metastases or any other tumours. The patient currently attends for regular clinical follow-up and has, at present, no signs of recurrence.

### *Case 2*

A 42-year-old female underwent wide excision (with a 2 cm border) of a pigmented lesion in the sacral region in 1966. Histopathology revealed a superficial spreading melanoma which was later microstaged, by rereading the original slide, as Clark level IV with a Breslow thickness of 1·75 mm (Fig. 3). At the time of excision of the primary CMM no evidence of regional lymph node or distant metastases was observed. In the following years she had regular examinations and after 10 years was discharged from further follow-up as presumably cured.

In 1984 she noticed a firm subcutaneous nodule on her right upper arm, and enlarged lymph nodes in her right groin. The nodule on her right arm was excised and a biopsy was taken from the inguinal tumour mass which, by ultrasound, was found to extend up to the parailiacal region. Histopathology revealed both lesions to be melanoma metastases (Fig. 4). A careful investigation failed to disclose further melanoma metastases or the presence of another primary melanoma.

A high dose DTIC monochemotherapy regimen (Pritchard *et al.*, 1980) was instituted and continued for 6 months, but this did not prevent further local progression of the inguinal tumour mass. Currently, she is receiving recombinant leukocyte-$\alpha 2$ interferon.

## DISCUSSION

The two female patients described with primary CMM who both experienced recurrence of their disease 18 years after diagnosis, surgical treatment and presumed cure, confirm the rare, but known, phenomenon of late recurrence of CMM (after more than 10 years). To the best of our knowledge and according to a recent review of the English literature, delayed occurrence of CMM metastases has been reported in only 12 well-documented cases (Koh *et al.*, 1984).

Delayed appearance of CMM metastases years after the primary tumour had been excised suggests that tumour cells had been present and held in check by host (probably immune) defence mechanisms (Spitler & Scott, 1982). Changes in the immune system of the host, e.g. killer cell activity, or changes in the metastatic potential or the immunogenecity of the resting tumour cells, e.g. by alteration of tumour antigens, might be possible reasons for the occurrence of delayed metastases (Koh *et al.*, 1984).

The clinical characteristics of the primary CMM, and the Breslow thickness—which is generally accepted as being the most important prognostic parameter in CMM—seem to be of minor importance in predicting the outcome of late CMM metastases. Early death (within 2 years) from melanoma occurs primarily in patients with thick ($>3·5$ mm) primary CMM, whereas early death from thin melanomas is rare. According to Day *et al.* (1983), deaths from melanoma after 5 years occur exclusively in patients who had intermediate thickness (1·7–3·4 mm) primary lesions. Our cases, and virtually all the other documented cases of delayed metastases of CMM, had thin to intermediate primary CMM lesions.

This raises the important question of whether the clinical phenomenon of delayed metastases of CMM has implications for the management and follow-up of CMM patients in general. Due to the statistically well-documented rising incidence of CMM (Balch *et al.*, 1985; MacKie, 1983), we may see more cases of delayed metastases in the future. We, like others (Koh *et al.*, 1984), therefore believe that even the few cases of delayed metastases of CMM observed so far justify an extension of the follow-up period of CMM patients beyond 10 years after primary excision and diagnosis.

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# Neonatal hypernatraemia in two siblings with Netherton's syndrome

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## SUMMARY

We report two siblings with Netherton's syndrome who developed hypernatraemia during the neonatal period. Although this is likely to have been due to trans-epidermal water loss in erythrodermic infants rather than to Netherton's syndrome specifically, this complication should be remembered in erythrodermic infants as a preventable cause of neonatal morbidity.

In 1949, Comel described a female with erythematous, serpiginous, migratory plaques, mainly on the trunk and extremities, which had a characteristic double-edged scale at the margin of the erythema, an entity he called ichthyosis linearis circumflexa (ILC). In 1958, Netherton described a child with a hair shaft defect (bamboo hair) which he considered to be a variant of trichorrhexis nodosa, pruritus and congenital ichthyosiform erythroderma (CIE). Subsequently, in 1964, Wilkinson, Curtis and Hawk suggested the name trichorrhexis invaginata for this hair defect. Netherton's syndrome has since come to indicate the triad of the hair shaft defect, an atopic diathesis and either ILC or CIE (Wilkinson, Curtis & Hawk, 1964; Altman & Stroud, 1969), probably with autosomal recessive inheritance (Greene & Miller, 1985).

In addition to these diagnostic criteria, some patients have been noted to have aminoaciduria, mental deficiency, neurological defects, delayed growth, recurrent infections and hyper- or hypogammaglobulinaemia. We report two brothers with Netherton's syndrome who had hypernatraemia in the neonatal period, a complication not previously described in this condition.

## CASE REPORTS

### *Case 1*

A Caucasian male was born by normal vaginal delivery at term following an uneventful pregnancy. His elder brother was entirely normal. At birth he weighed 3·6 kg and was noted to

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have a generalized erythroderma with exfoliation, but was otherwise normal. Over the next few days he received routine neonatal care (he was not placed on an infant warming pad) and continued to do well though his skin stayed dry, erythematous and scaly. However, by 10 days of age he was noted to be dehydrated and irritable despite having breast fed well and not having had diarrhoea. General examination was normal and culture of blood, urine and CSF produced no growth. Serum electrolytes showed sodium 160 mmol/l with normal potassium, chloride and urea. Urinary specific gravity was 1·027. A tentative diagnosis of dehydration secondary to trans-epidermal water loss was made, and there was a good response to rehydration, his subsequent course being without incident.

At the age of one year, he was seen at the Dermatology Department at the University of Colorado, having had continued problems with red scaly skin. On examination, he was found to have erythematous skin with fine scale over his trunk and limbs and thick lamellar type scale over his scalp and buttocks. His hair was short, sparse and twisted but he had normal teeth and nails. Microscopic examination of his scalp, eyebrow and body hair showed the typical ball and cup deformity of trichorrhexis invaginata, and a diagnosis of Netherton's syndrome was made. Since that time he has remained erythematous and scaly with a recurrent eczema-like eruption, necessitating therapy with emollients, intermittent topical steroids and occasional hospital admission for skin care. He has otherwise remained well, exhibiting normal growth and development.

*Case 2*

A Caucasian male and brother of Case 1, was born by normal vaginal delivery at 36 weeks gestation, after an uneventful pregnancy. He weighed 3 kg, and was noted at birth to have erythematous dry peeling skin. He received normal neonatal care (he also was not placed on a warming pad), but at 48 h, in addition to his persistent skin condition, he was noted to be dehydrated, cyanotic and in respiratory distress. There were no signs of infection, and he had not had diarrhoea. Blood gases at that time showed a pH of 7·47, a $pCO_2$ of 2·1 KPa and a $pO_2$ of 6·6 KPa. Serum electrolytes showed sodium 182 mmol/l, potassium 5 mmol/l and chloride 106 mmol/l. He was treated with antibiotics and intravenous fluids. However, over the next few hours he continued to deteriorate, and developed grunting respirations; he was noted to have a 4 cm palpable liver, and a 2/6 systolic murmur was heard over the right costal area. A tentative diagnosis of pulmonary outflow tract obstruction with a closing ductus arteriosus was made. His condition continued to deteriorate despite a prostaglandin infusion and he suffered a cardiorespiratory arrest, but was successfully resuscitated with volume replacement and sodium bicarbonate. He was transferred to Denver Children's Hospital for further evaluation and care.

On arrival at Denver, examination revealed a cyanotic infant with a 2/6 systolic murmur, maximal at the right upper sternal area, and generally diminished pulses. He was clinically thought to be dehydrated, and serum electrolytes showed sodium 187 mmol/l with chloride and potassium within the normal range. Urinary specific gravity was 1·029, urinary sodium was within normal limits, and there was no glycosuria. The high serum sodium fell with rehydration to 150 mmol/l over the subsequent 72 h. Cardiological investigation revealed pulmonary stenosis/atresia and a hypoplastic left heart, and on day 6 he underwent insertion of a Blalock-Taussig shunt with pulmonary valvotomy. However, he did poorly post operatively and finally died on day 8.

Dermatological examination prior to surgery revealed an infant with generally dry, erythrodermic skin with fine scale over the trunk and thick scale over the scalp. He had short blond hair, microscopic examination of which showed mainly vellous hair and a few terminal hairs, one of which showed a fracture through a node, although a definite ball and cup deformity could not be identified. In view of this and the history of his brother's condition, a diagnosis of probable Netherton's syndrome was made.

## DISCUSSION

The presence of a lamellar-type ichthyosis, trichorrhexis invaginata, and an eczematous eruption in Case 1 makes a diagnosis of Netherton's syndrome likely. The second child also had an ichthyosiform erythroderma and abnormal hair at birth and it was therefore considered that a diagnosis of Netherton's syndrome was also justified.

Hypernatraemia in the neonatal period is an uncommon finding, and the occurrence of this in two siblings is even more so. Furthermore, neither of the infants had any of the recognized causes of neonatal hypernatraemia. Specifically, neither was significantly premature, hypernatraemia with subsequent hyponatraemia being recognized in premature infants born between 24 and 30 weeks gestation. Neither of the children had had diarrhoea, and neither were found to have polyuria, which, taken in conjunction with urinary specific gravities showing concentrating ability, makes a diagnosis of diabetes insipidus unlikely. The possibility does exist that the hypernatraemia in the second child may have been due in part to the administration of sodium bicarbonate, although this cannot have been the sole cause as hypernatraemia was noted prior to the sodium bicarbonate infusion.

It is possible that the hypernatraemia is somehow connected with Netherton's syndrome, although the mechanism is not clear. Theoretically, it could be due to abnormal renal sodium handling, as patients with Netherton's syndrome have been reported to exhibit aminoaciduria. However, the fact that both children were able to concentrate their urine, and that the second child was found to have normal urinary sodium does not support this hypothesis. It has been suggested that the aminoaciduria in Netherton's patients is a result of steroid therapy rather than being due to a specific renal problem (Greene & Miller, 1985). A more likely possibility is that the hypernatraemia is not related specifically to Netherton's syndrome, but that neonates who are erythrodermic simply suffer excessive trans-epidermal water loss, which, if not recognized and replaced with suitable fluids, may lead to hypernatraemia. If this is the case, then the use of infant warming pads in these erythrodermic infants may compound the problem by increasing insensible trans-epidermal water losses. However, if this hypothesis is correct, the question arises as to why hypernatraemia is not reported more often in neonates who are erythrodermic for any reason, not just as a result of Netherton's syndrome.

We report these two cases of neonatal hypernatraemia with Netherton's syndrome to alert the clinician of this cause of neonatal morbidity. Early recognition of hypernatraemia and prompt institution of corrective fluid and electrolytes may prevent avoidable neonatal morbidity in these erythrodermic neonates.

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### Cutaneous leishmaniasis

MADAM, We read with interest the recent letter from Dr Vella Briffa (*British Journal of Dermatology*, 1985, **113**, 370) in which he gave an historical account of cutaneous leishmaniasis in the Maltese islands. Dr Vella Briffa described his attempts to culture leishmania from nine of the 13 patients in his series but, like all his predecessors, he was unsuccessful.

In March 1985, a 71-year-old English gentleman was referred to the Cambridge Military Hospital with several inflamed, granulomatous lesions on his face. These lesions had first appeared in December 1984 following a holiday on the island of Malta in September of that same year. In the 10 years prior to his referral this patient had not visisted any other leishmanial endemic area nor had he visited any of the smaller islands around Malta. A Montenegro skin test was positive and histological examination of tissue taken from the edge of one of the lesions revealed an intradermal granulomatous infiltrate, with intracellular amastigotes identified in Giemsa stained sections. Culture of part of the biopsy specimen in biphasic Evans-modified Tobie's medium (Evans, 1978) was successful and isoenzyme typing by the thin-layer starch gel electrophoresis method of Godfrey & Kilgour (1976) modified by Evans *et al.* (1984) using 12 enzymes, has shown the infecting organism to be *L. donovani infantum* London zymodeme 49. This leishmanial strain has been allocated the WHO code MHOM/MT/85/BUCK.

The patient, who was not keen to have treatment with intravenous sodium stibogluconate, was referred to the Hospital for Tropical Diseases where he was treated successfully with topical applications of an ointment containing paromomycin in benzylkonium chloride. Paromomycin is an aminoglycoside derivative from *Streptomyces rimosus* and its successful use in the therapy of cutaneous leishmaniasis was first reported in animal experiments (El-On *et al.*, 1984), and more recently in human subjects (El-On *et al.*, 1985).

Accurate taxonomic identification of *Leishmania* isolated from cutaneous lesions can be extremely important from a therapeutic point of view (Peters, Evans & Lanham, 1983) and is always important epidemiologically. This is the first case of primary cutaneous leishmaniasis caused by *L. donovani infantum* to be reported from Malta, and our findings in this patient complement those of Rioux *et al.* (1980) and Bellazoug (1982) in showing that this leishmanial sub-species, previously considered to cause only systemic disease, is capable of causing primary cutaneous disease.

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ACKNOWLEDGMENT

We are grateful to Dr Michael Johnson, Consultant Dermatologist, Epsom District Hospital, for permission to report this case.

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## Reply

MADAM, The isolation by Drs Jolliffe, Evans and Bryceson of *L. donovani infantum* from cutaneous leishmaniasis acquired in Malta is an important step in unravelling the epidemiological situation there. It now seems even more important to determine whether all indigenous cases are due to the same subspecies which causes visceral disease, and if so, whether the altered ratio of cutaneous to visceral disease reported recently is due simply to increased surveillance or to a modified host/parasite relationship.

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## Herpetiform napkin dermatitis: napkin dermatitis simulating an acute herpes simplex infection

MADAM, We wish to report an eruption of the napkin area, closely resembling an acute herpes simplex infection, as it is important to be aware than an irritant napkin dermatitis may present in this way.

An 8-month-old boy was referred to the Dermatology Department of the Leicester Royal Infirmary in October 1984. His parents reported that for several days he had been devloping blisters in the napkin area. Apparently, he had had an identical eruption 4 months previously.

On examination there was a profuse eruption of vesicles and pustules, each on an erythematous background. Some of the lesions appeared to have burst leaving shallow erosions. The clinical appearance was strongly suggestive of acute herpes simplex infection (Fig. 1). However, repeated swabs for virus culture were negative, as were bacterial and fungal cultures. He was treated with topical povidone-iodine ointment.

Lesions continued to appear over the course of the next 3 weeks and the child was therefore admitted to hospital. The napkin area was left exposed and all the lesions healed within a few days. After discharge he remained well with only a few minor lesions in the napkin area until March 1985 when a further severe eruption appeared, identical to that seen previously. A skin biopsy was performed which showed parakeratosis and a large intraepidermal vesicle, but no viral inclusions. There was some non-specific dermal infiltration. Cultures were again negative for viruses, bacteria and fungi.

Napkin eruptions are very common. The most frequent are thought to be irritant in origin. It is not known precisely what mechanisms are involved in this process, although ammonia, urine, bacteria,

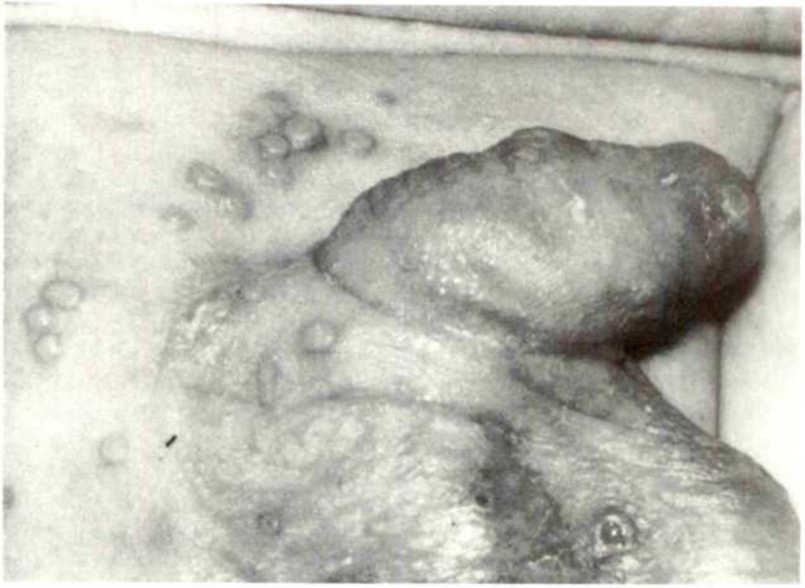

FIGURE 1. Several herpetiform lesions are clearly seen in the pubic region and on the scrotum.

*Candida*, stools, detergents, friction and occlusion are often put forward in varying combinations as likely offenders. Vesicles are a documented accompaniment of napkin dermatitis (Montes, 1978) and the pathological changes seen in vesicular napkin dermatitis were described in detail as long ago as 1908 (Ferrand, 1908).

It does not appear to be recognized widely that there may be real diagnostic confusion between an irritant dermatitis of the napkin area and an acute herpes simplex infection, and this point is not made in the standard texts of the subject. The problem is mentioned, however, in Verbov and Morley's *Colour Atlas of Paediatric Dermatology* (1983). They have published a photograph of just this type of eruption with the legend: 'Napkin dermatitis herpetiform type. These vesicles rapidly dried up. No organism grew on culture.' (Verbov & Morley, 1983).

We are reporting this case, therefore, to draw attention to the fact that an irritant napkin dermatitis may, on occasion, present very acutely with profuse vesicles and closely resembling an acute herpetic infection. This possibility should be borne in mind when the time course of an apparently typical herpetic infection is protracted, or when viral cultures are negative.

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## Heritability of Behçet's disease

MADAM, A recent paper by Dündar, Gençalp and Şimşek (*British Journal of Dermatology*, 1985, **113**, 319) reports seven families with Behçet's disease as evidence for the invovlement of genetic factors in the pathogenesis of the disease. In spite of the evidence for an immunogenetic background provided by the association of HLA antigens with the disease in different ethnic groups (Lehner, Welsh & Batchelor, 1982; Okuyama *et al.*, 1984), no investigations on heritability have been reported, probably because familial cases of Behçet's disease are very rare. Further information on the screening procedures used and the size of the population examined by Dundar *et al.* could provide more insight into the heritability of the disease.

However, environmental factors could also play an important role, as would be expected from a multifactorial model of inheritance, and this could explain some of the familial clustering which might be attributable to the shared environment. Kudwah and Madkour (1985) observed the disease in unrelated spouses of Behçet's patients and viral involvement has been claimed to play an important aetiopathogenetic role by several authors (Eglin, Lehner, Subak-Sharpe, 1982; Efthimiou *et al.*, 1984).

We have carried out a prevalence association study on HLA and Behçet's disease in 37 Italian patients (27 with the complete syndrome and 10 incomplete, all with ocular involvement). We also carried out a careful clinical examination of first degree relatives of 10 patients (35 siblings and 19 parents) and collected anamnestic data from the remaining 27 patients. Among the relatives examined, three siblings had recurrent oral ulcers, and one of them also had recurrent bronchopneumonia, without any other sign of the disease. Among the relatives of the remaining patients, one case of the complete syndrome was reported.

We would emphasize that both environmental and immunogenetic factors seem to be required for the manifestation of the disease, which is in accordance with the multifactorial model generally accepted for HLA associated diseases. Thus, a correct estimation of the risk for patient's relatives in a given population could only be drawn from a wide epidemiological study.

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## Reply

MADAM, Drs Sensi and Baricordi say that 'In spite of the evidence for an immunogenetic background provided by the association of HLA antigens with the disease in different ethnic groups, no investigations on heritability have been reported, probably because familial cases of Behçet's disease are very rare'. I think this is incorrect, because there are reports about the heritability of Behçet's disease from several different countries (Andaç, Pamukçu & Ergüllü, 1979; Aronsson & Tegner, 1983; Berman, Trappler & Jenkins, 1979; Yürütken, Yilmazer & Özkarakas, 1979).

We would also like to report two more families affected by the disease, further to the seven already reported. In one family the grandmother (aged 60), a daughter (aged 30) and a granddaughter (aged 9) have the disease; in the second, the father (aged 56) and daughter (aged 26) were affected. In the first family, two other granddaughters (aged 5 and 7) have oral aphthae; in the second, another daughter (aged 16) had oral aphthae. There is no consanguinity in any of these nine families.

Chromosome studies have been performed in a group of Behçet's patients (not in the familial cases) but no specific changes have been shown.

It was suggested that environmental factors might be responsible for the occurrence of the disease. I agree that environmental factors could be important; however, our cases have other siblings and close relatives living in the same house or close by who do not have the disease. Also, we have no case of a spouse with Behçet's disease. We have now recorded more than 300 cases from different parts of Turkey; apart from the nine families with more than one member affected, all the remaining cases are sporadic.

HLA-B5 is found in 37·5% of the Turkish population, but occurs in 68 4% of cases of Behçet's disease in Turkey (Dündar & Özerkan, 1980). Thus, the HLA-B5 tissue type appears to be a risk factor for the development of Behçet's disease. It is possible that a slow virus (as yet unidentified) may cause the disease in individuals of HLA-B5 tissue type. There might also be a genetic predisposition to infection with such a virus.

Aransson and Tegner (1983) describe Behçet's disease in two brothers who lived separately all their lives.

Some studies have suggested a recessive, and others a dominant, mode of inheritance of the disease. A hereditary factor, possibly linked to the immune system, could explain the tendency to develop the disease, which might be precipitated by infection with a virus or by environmental factors. The report of the disease in spouses was probably fortuitous.

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## Liver ultrasound screening during methotrexate treatment

Madam, We also use ultrasound as a screening procedure during methotrexate treatment of psoriasis (*British Journal of Dermatology*, 1985, **113**, 699) after a patient died as an indirect result of a follow-up liver biopsy.

Although we recommend an initial liver biopsy prior to methotrexate treatment for all patients requiring long-term therapy, follow-up biopsies are then only recommended (and then probably every 3 years) on patients who develop abnormal ultrasound scans or who have risk factors such as persistantly raised liver enzymes (including gamma glutamyl transferase), concommitant medical conditions predisposing to liver disease, or an abnormal pre-treatment liver biopsy.

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*British Journal of Dermatology* (1986) **114**, 750.

# *News and Notices*

## Incontinentia Pigmenti Association

This group has been formed by a group of patients and parents. Further details can be obtained from Mrs Jean Palmer, Secretary, 98 Dynes Road, Kemsing, Sevenoaks, Kent TN15 6RF, U.K. Telephone Otford (09592) 3779.

## Forthcoming Course

Practical Dermatology Course for Registrars and Senior Registrars will be held on 30 and 31 October 1986. Further details are available from Dr F.Wojnarowska, Dermatology Department, The Slade Hospital, Headington, Oxford OX3 7JH, U.K.

## 'Workgroup Skin': A Bi-Annual Newsletter

For some years, most of the investigators in the Netherlands who are concerned with basic or applied skin research have belonged to an informal association called 'Workgroup Skin'. This group is now publishing a newsletter; the first issue was for March 1986 and it is hoped that future issues will appear at 6-monthly intervals. Copies may be obtained free of charge by writing to the Secretary: Dr F.W.Bauer, Department of Dermatology, University of Nijmegen, 6524 MJ Nijmegen, The Netherlands.

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| kilogram(s) | kg | centimetre(s) | cm | millilitre(s) | ml |
| milligram(s) ($10^{-3}$g) | mg | second(s) | s | gravitational acceleration | *g* |
| microgram(s) ($10^{-6}$g) | μg | cubic millimetre(s) | $mm^3$ | micrometre(s) | μm |
| nanogram(s) ($10^{-9}$g) | ng | millimetre(s) | mm | per cent | % |
| picogram(s) ($10^{-12}$g) | pg | millicurie(s) | mCi | isotopic mass number placed as | $^{131}$I |
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